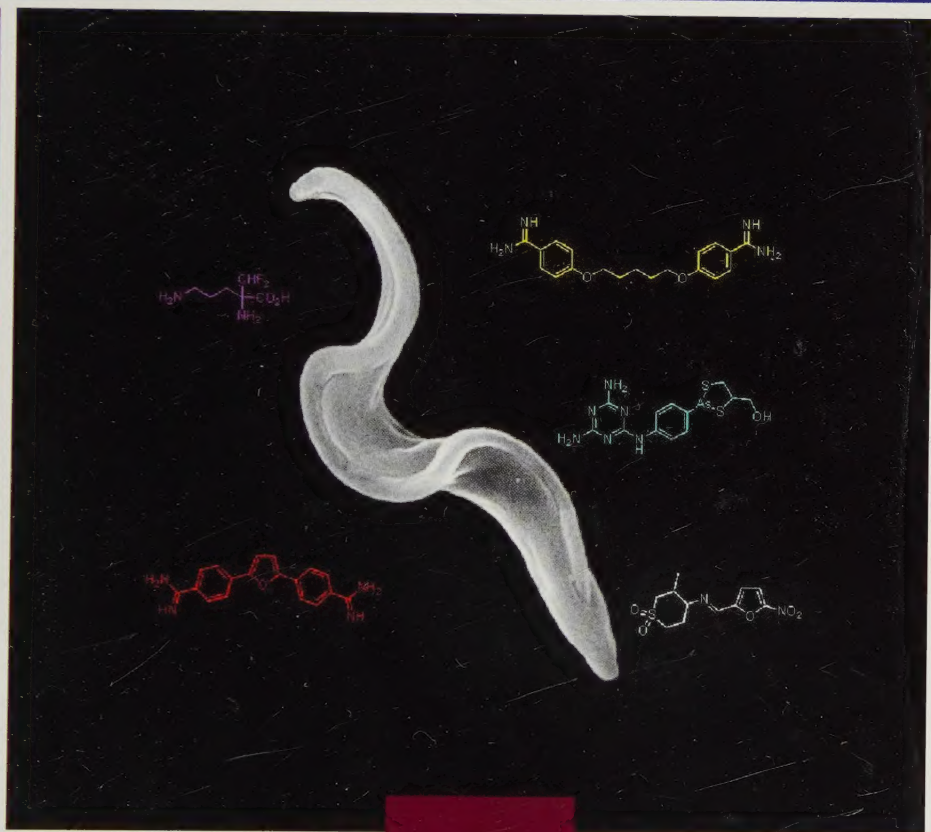


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Preface

The volume opens with a detailed overview of the phylogenetic analyses of parasites by David Morrison of the National Veterinary Institute and Swedish University of Agricultural Sciences. The introduction of sequence data as a source of phylogenetic information has been accompanied by a surge of interest and activity to reconstruct the phylogenetic history of life on earth. Volume 54 of *Advances in Parasitology*, which was a special volume, was focused on a phylogenetic perspective of the evolution of parasitism. This review gives a personal view concerning important contemporary issues in phylogenetic analyses. It is likely to be of value to all those interested in constructing phylogenetic trees as it aims to unravel some of the complexities of phylogenetic analyses and critically examines various topics such as homology versus analogy, rooted versus unrooted trees, bootstrap values and choice of outgroups. The author goes on to consider the strengths and weaknesses of various evolutionary models and uses a real molecular data set of *Cryptosporidium* to illustrate the points raised.

Human trypanosomiasis is spreading again in Africa and new drugs are urgently required. The contribution by Michael Barrett of the Institute of Biomedical and Life Sciences at the University of Glasgow, UK, and Ian Gilbert of the School of Life Sciences of the University of Dundee, UK, reviews recent studies investigating the pathways of nutrient uptake by trypanosomes in order to selectively carry toxins into their bodies.

Many different plasma membrane transporters have been identified in trypanosomes and these are outlined; the aminopurine (P2) transporter appears to be a significant route of entry for at least two trypanocidal drugs. Other possible routes are via other purine.

pyruvate, hexose, lipid, and amino acid transporters and possibly by endocytosis. The recent mapping of the genome of *Trypanosoma brucei* should help to identify new transporter systems to introduce trypanocidal drugs into the interior of trypanosomes.

Patrick Skelly at the Tufts Cummings School of Veterinary Medicine, North Grafton, USA and Alan Wilson at the Department of Biology, University of York, UK also explore the outer surface (including the plasma membrane) but of very different parasites, the schistosomes. The outer surface (tegument) is bounded by a plasma membrane overlain by a secreted membranocalyx to which host molecules can associate and promote parasite survival by immunoevasion, so that adults can survive in the bloodstream of humans for many years. The authors review the molecular organizations and biochemical functions of parasite surface proteins and glycans; while the involvement of many surface enzymes in the surface complex is shown by proteomic analysis.

Rafael Toledo and José-Guillermo Esteban of the University of Valencia, Spain together with Bernard Fried at Lafayette College, USA review the literature on pathological and immunological effects of infection by intestinal trematodes in their definitive hosts. In the past, much of this information has been scattered in the literature and the authors have succeeded in compiling a comprehensive review that brings together a number of common facts and features associated with trematode infections. In contrast to studies on nematodes, surprisingly little is known about the pathological and immunological changes associated with intestinal trematode infections. Particular emphasis is given to species from six selected families that are of medical and/or veterinary importance. It is clear that several factors associated with the species of both host and parasite may influence the course of the infection and resulting pathology. There is much of interest here and the review highlights areas where further research is required.

Edoardo Pozio of the Istituto Superiore de Sanità, Rome, Italy and Darwin Murrell at the Royal Veterinary and Agricultural University, Frederiksberg, Denmark review the systematics, ecology and epidemiology of trichinellosis. Recent molecular studies have revolutionized the systematics of the genus *Trichinella* and this has resulted in its

being divided into two clades, one including at least five species in which the larvae form capsules in the tissues and another three species without larval capsules. Some species have recently been found to parasitize reptiles rather than mammals. Much new information on the phylogeny, ecology and geographical distribution of these species is reviewed and may have great value for the understanding of the evolutionary biology of other parasitic helminths and may also increase the value of this genus as a model for research on parasitism in general.

J.R. Baker
R. Muller
D. Rollinson

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ABSTRACT

Phylogenetic analysis has changed greatly in the last decade, and the most important themes in that change are reviewed here. Sequence data have become the most common source of phylogenetic information. This means that explicit models for evolutionary processes have been developed in a likelihood context, which allow more realistic data analyses. These models are becoming increasingly complex, both for nucleotides and for amino acid sequences, and so all such models need to be quantitatively assessed for each data set, to find the most appropriate one for use in any particular tree-building analysis. Bayesian analysis has been developed for tree-building and is greatly increasing in popularity. This is because a good heuristic strategy exists, which allows large data sets to be analyzed with complex evolutionary models in a practical time. Perhaps the most disappointing aspect of tree interpretation is the ongoing confusion between rooted and unrooted trees, while the effect of taxon and character sampling is often overlooked when constructing a phylogeny (especially in parasitology). The review finishes with a detailed consideration of the analysis of a multi-gene data set for several dozen taxa of *Cryptosporidium* (Apicomplexa), illustrating many of the theoretical and practical points highlighted in the review.

1. INTRODUCTION

In many ways, attempting to reconstruct the phylogenetic history of a group of organisms is the hardest thing that a biologist can try to do. This is because the patterns being examined are unobservable historical ones rather than contemporary ones that can be directly examined and experimentally tested. Consequently, the only protection that we can have against false conclusions is the quality of the data and the

data analysis—there are no alternative experimental procedures that can be used to independently verify the results, except the collection of different data and the performance of different analyses. A phylogenetic analysis is thus only as good as the steps taken to ensure the highest quality of data and to evaluate and use the most appropriate mathematical model for the data analysis.

Phylogenetic analysis may be complex, but it is still relatively straightforward in most cases—it just requires a little bit of care and some attention to the appropriateness of the various methods in the context of the data to be analyzed. Unfortunately, in some areas of biology, including parasitology, overly simplistic analyses still seem to be the order of the day for many practitioners. In the modern world, however, with the advent of more realistic models of character evolution, phylogenetic analyses can no longer be treated as “black boxes” into which data are fed and from which a tree spontaneously emerges. Practitioners need to be aware of what they can put into the analysis and how to interpret what comes out, as well as how best to choose an appropriate analysis.

In this review, I therefore provide an update on some of the themes that seem to me to be the most important contemporary issues in phylogenetics. This will give you some idea of where we have come in the last 10 years or so, and where we might go in the next 10. The review is by no means comprehensive in scope, if only for reasons of space, nor do I describe any of the actual methods in detail. Instead, I assume that the reader has some familiarity with phylogenetic analysis in a general sense. There are a number of excellent books and journal reviews that provide a more general introduction to the topic than I am undertaking here. These include the review articles of Morrison (1996), Swofford *et al.* (1996), Whelan *et al.* (2001) and Holder and Lewis (2003) as well as some introductions specifically for “gene jockeys” (e.g. Baldauf, 2003; Delsuc *et al.*, 2005) and parasitologists (Stevens and Schofield, 2003). There are also excellent books, varying from the introductory (Salemi and Vandamme, 2003; Hall, 2004) to the detailed (Nei and Kumar, 2000; Felsenstein, 2004). I will assume that the reader is at least tolerably familiar with one or more of these works.

Instead, I am going to provide a detailed consideration of those topics that seem to me to be important issues for one of two possible

reasons: either they are clearly misunderstood by many non-experts, or they are going to become increasingly important as the new millennium progresses. In the first category, I include topics such as homology versus analogy, rooted versus unrooted trees, bootstrap values and the choice of outgroups. In the second category, I include sophisticated evolutionary models, including detailed model evaluation and tree-building that explicitly uses these models. These are topics that are incompletely covered in most of the works cited above, and so it is worthwhile to review them here. I also provide a detailed analysis of a real molecular data set, which neatly illustrates in a practical sense most of the theoretical points made. The major topic that I have (mostly) avoided in this review is that of sequence alignment, which would require a separate paper of its own.

Throughout this paper I am going to refer to the objects being analyzed as taxa (singular taxon). However, in any particular analysis these objects might in reality be species, or they might be higher-level taxonomic categories such as genera or families, or they might be other categories such as populations, individual organisms or single copies of a multi-copy gene family within an individual. Moreover, my discussions will be centred on the analysis of molecular data, this being currently the most commonly used form of data in parasitology (as opposed to the more traditionally used macro-biological characteristics, such as host, tissue and vector specificities). However, most of the points that I make will apply to other forms of data as well.

2. SOME BASICS

2.1. Random and Systematic Error

There are two distinct types of error that will affect a phylogenetic analysis: (i) random or stochastic error, and (ii) systematic error. These topics will recur throughout this review and so it is worthwhile highlighting them here.

Stochastic error is error that results from sampling. That is, we cannot make a complete inventory of all of the data that could be collected to address any one scientific question, and so we collect a sample

instead. That sample may or may not be representative of the complete collection of data, and this results in random error. Systematic error, on the other hand, results from mismatches between our goal and our sampling and analytical procedures. That is, we may (unintentionally) collect data from taxa that are inappropriate (e.g. diseased), or choose to analyze the data with an inappropriate evolutionary model. Systematic error is thus associated with the accuracy of our answer (i.e. how close to the truth we get), while random error is associated with the precision with which we can present that answer (i.e. how repeatable it is). In a phylogenetic study, random error is always expected to occur but we can attempt to reduce its impact, while systematic error is something that we actively strive to avoid if we can.

Random error is usually quantified by some measure of support for the branches of the phylogenetic tree, as discussed in Sections 3.3–3.5. In these cases, a large amount of “support” does not indicate the accuracy of the tree, since the measure refers to random error rather than to systematic error. We can try to reduce the impact of random error by increasing the number of characters on which we base our tree, as discussed in Section 4.1. It may also be worth distinguishing two types of random error (DeBry, 2005): sampling error, where the “true” tree for a finite data set is not the same as that for an infinite data set for the same taxa; and tree-search error, where the tree found by the search algorithm for a finite data set is not the optimal tree for that data set. Increasing the number of characters should help reduce both types of error, but our choice of tree-building method may also help with the latter, as discussed in Section 6.

Systematic error cannot usually be quantified, as it results from the fact that some non-historical signals in our data are of the same magnitude as the historical signals, and we cannot know which are which. We can try to reduce the impact of systematic error by our choice of taxa (Section 4.1), by our choice of evolutionary model (Section 5) and by our choice of tree-building method (Section 6).

2.2. Homology and Analogy

It seems to me that one of the most obvious problems in phylogenetic analysis is the tendency of researchers to re-invent the wheel periodically,

by not realizing that the problem they have just encountered is merely a specific example of a more general problem that has been known for centuries. Systematists have been dealing with phylogeny reconstruction for 150 years now, and certain general principles have emerged – many contemporary problems have appeared simply because non-systematists (e.g. molecular biologists) have ignored these principles, either explicitly or implicitly, and then had to re-discover the principles for themselves.

The most egregious example is the tendency of many researchers to equate the words “similarity” and “homology”. While the term homology has been used historically to refer to a wide variety of concepts (Sluys, 1996; Butler and Sidel, 2000), the evolutionary concept of homology refers to the relationships of features that are shared among taxa due to common ancestry (i.e. they all inherited the feature from their most recent common ancestor). Systematists have long insisted on this definition, and there have been calls for all molecular biologists to use it as well (Reeck *et al.*, 1987). The point is that in phylogenetic analysis the definition serves the very useful purpose of highlighting the fact that similarity \neq homology. In phylogenetics (and comparative biology in general),

$$\text{similarity} = \text{homology} + \text{analogy}$$

instead. Analogy refers to similarity resulting from the same function rather than similarity resulting from the same evolutionary origin. Analogy will lead to incongruences among the characters compared to the relationships shown by homology, and these will confound our ability to detect homology. That is, some of the apparent relationships among taxa will be due to homology and some will be due to analogy, and these two patterns of relationship are unlikely to be in agreement. We are then caught in the bind of trying to disentangle the two patterns, because the one due to homology is the one that we really want, in an evolutionary context.

Analogy results from: (i) parallelism, where the same character state arises in separate evolutionary lineages; (ii) convergence, where superficially similar character states arise in separate lineages; and (iii) reversals, where a derived character state reverts to the ancestral state. Together, character incongruences caused by these processes are called homoplasy. In molecular biology, gene duplication is one

example of parallelism, functional similarities leading to protein structural similarity without sequence similarity are examples of convergence, and multiple nucleotide substitutions are examples of reversal. Such processes are known to be quite common in molecular evolution (Dickinson, 1995; Fitch, 2000), especially in primary nucleotide sequences, so that they cannot be ignored if phylogenetic tree-building is to be successful. It sometimes turns out that other molecular features produce less homoplasy (e.g. Rokas and Holland, 2000; Gough, 2005), but the initial enthusiasm engendered for such markers frequently wanes after more extensive data are collected and homoplasy then becomes obvious.

The bottom line is that many of the problems in phylogenetic analysis of molecular data are caused simply by ignoring analogy, either explicitly or implicitly. For example, when referring to a database search as a "homology search" (which is what the researcher would like it to be) rather than a "similarity search" (which is what it is in practice), failures of the search will result from detecting analogy instead of homology (as well as failure to detect homology, of course). From this point of view, database similarity searches must always fail to some extent because similarity does not arise solely from homology. Similarly, when performing a multiple sequence alignment by maximizing the similarity among the sequences (which is what almost all of the computer programs do), all failures of the alignment will be due to aligning analogous segments of sequence instead of homologous ones. From this point of view, sequence alignment by similarity must always fail to find the true evolutionary alignment because homology is not the cause of all observed similarity (even if it is the most common cause).

Finally, when building a phylogenetic tree by similarity, which is what all of the programs do at heart, since they assume that identical sequence segments represent historical relationships, all failures will be due to mis-interpreting analogy as homology. This particularly applies to tree-building methods based explicitly on genetic "distances," as these methods try to maximize the overall similarity among the taxa on the tree, and this methodological approach can only successfully recover the true evolutionary tree if the observed similarity among the taxa arises solely from homology. Character-based methods, such

as parsimony and likelihood, try to minimize the amount of inferred homoplasy on the tree, as part of their optimality criterion, which gives them a theoretical advantage (and one that also appears in practice).

From this point of view, many currently recognized practical problems, such as long-branch attraction and compositional biases, are merely specific examples of how analogy appears in molecular biology. Analogy will create convergences and parallelisms, and these will confound the attempt to detect homology. So reconstructing evolutionary history using molecular biology is *a priori* neither better nor worse than using any other source of data, because the same limitations apply. However, the expectation is that it should be easier to assess homology using molecular sequence data than it is for morphological data and some other types of molecular data (Freudenstein, 2005), and this is partly why sequence data have become so popular.

3. INTERPRETING TREES

3.1. Rooted versus Unrooted Trees

There seems to be some confusion in the applied literature (not just in parasitology) about how to interpret phylogenetic trees. In particular, the distinction between an unrooted tree and a rooted tree is often not made, or is wrongly made, leading to blatantly incorrect interpretations of the trees under discussion. For example, people sometimes write about “branching order” on a tree when the tree has no root, and yet it is the root that determines the order; or they write about “groups” or “clusters” of taxa on an unrooted tree, when it is the root that determines the groupings. I am therefore going to belabour the issue here, on the grounds that it is probably the most useful thing that I can do about it.

The important point to understand is that an unrooted tree has no evolutionary direction (i.e. from ancestors to descendants), while a rooted tree does. This conceptual distinction is not trivial, because it leads to all sorts of significant consequences in an evolutionary analysis.

In particular, phylogenetic theory makes it clear that the only groups of taxa with any meaning in an evolutionary context are groups that consist of all of the descendants of their most recent common ancestor. This being so, if the objective of an analysis is to recognize groups of taxa then this can only be done using a rooted tree—there is no meaningful way to interpret an unrooted tree in an evolutionary context. Thus, a rooted tree compared to an unrooted tree has: (a) ancestors and descendants; (b) a branching order in evolutionary history; (c) ancestral and derived character states; and (d) evolutionary groups. From a practical point of view, this means that almost all of the questions being asked by biologists, which they are trying to answer by performing a phylogenetic analysis, can only be answered using a rooted tree.

The confusion between rooted and unrooted trees may arise from the fact that most computer programs for phylogenetic analysis proceed by producing only an unrooted tree. The main reason for this is quite simple: there are far fewer unrooted trees than there are rooted trees, and so it is computationally more efficient to produce an unrooted tree first and then to add a root to it later. The mathematics of the situation are quite straightforward for bifurcating trees: each unrooted tree can be rooted on any one of its branches, and thus for any set of taxa the number of rooted trees equals the number of unrooted trees multiplied by the number of branches on the unrooted tree. An example is shown for five taxa in Figure 1. In this example, there is only one possible shape for the unrooted tree (as shown), but there are seven different rooted trees that can be derived from it, and these have two distinct shapes. Clearly, the situation becomes increasingly complex as the number of taxa increases—even for nine taxa there are only 135 135 unrooted trees compared to 2 027 025 rooted trees. It is therefore no surprise that computationally the best procedure is to get an unrooted tree first and then to root it afterwards.

This means that biologists must root the tree themselves (Bryant, 2001) rather than having it done automatically. This is usually done by providing an outgroup in the phylogenetic analysis, as discussed in Section 3.2. If a suitable outgroup is chosen then the unrooted tree should be able to be unambiguously turned into a rooted tree. This means that the phylogenetic tree now has an evolutionary direction. That is, an unrooted tree merely has a shape (or topology), but a

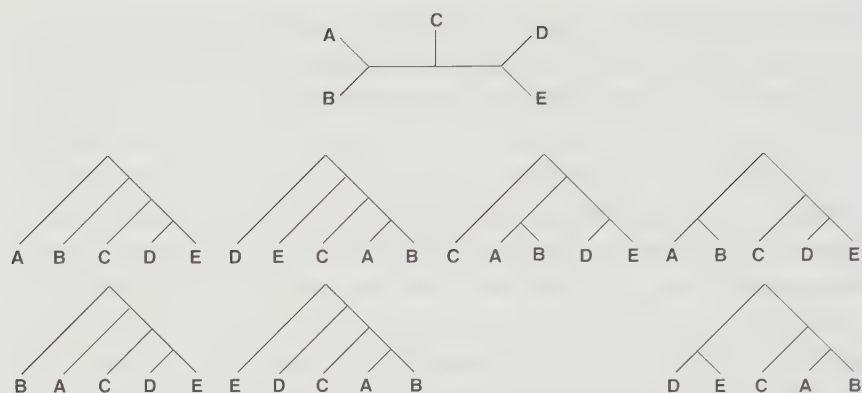


Figure 1 An unrooted tree for five taxa (top) and the seven rooted trees that can be derived from it (below). Each of the rooted trees (drawn with the root at the top) is rooted on a different one of the seven branches of the unrooted tree.

rooted tree has much more as a result of the inferred time direction. There are four closely related aspects that are worth emphasizing when considering a rooted tree as opposed to an unrooted one.

First, ancestors and descendants can be recognized. Clearly, the ancestors in most analyses are merely inferences (or hypotheses) derived from the analysis, while the final descendants are contemporary taxa. Therefore, the inferred ancestors and their characteristics are an important result from the analysis. This may not be true of population-level studies, however, where ancestral taxa can be contemporaneous with their descendants (e.g. parents and children occurring in the same sample).

Second, the branching order of evolutionary history can be determined. On an unrooted tree, without a time direction, we have no idea of what the branching order was, but on a rooted tree the branching order is made explicit. This should be obvious from Figure 1, where a single unrooted tree can yield seven different possible branching orders through time for the five taxa, only one of which can be correct. It is the rooting of the tree that determines which of the seven scenarios is the correct one.

Third, but equally important (Bryant, 2001), the direction of character-state changes can be determined. This means that ancestral

character states (i.e. those in the ancestors) can be distinguished from derived character states (those in the descendants). This information can be just as important for answering biological questions as is the branching order of the taxa, as the focus is now on the characters rather than the taxa. Indeed, in studies of molecular evolution this is often the primary information that is desired from the analysis.

Finally, evolutionary groups can be recognized on a rooted tree but not on an unrooted tree. An unrooted tree only indicates partitions (or splits) in the group of taxa. An example of this is shown for four taxa in Figure 2. There are three possible ways to split four taxa into partitions of two taxa each, and the unrooted tree in Figure 2 shows one of them, with taxa $\alpha + \gamma$ in one partition and taxa $\beta + \delta$ in the other partition. Thus, an unrooted tree contains information that allows us to eliminate possible groups from consideration. However, it does not contain positive information about groups because not

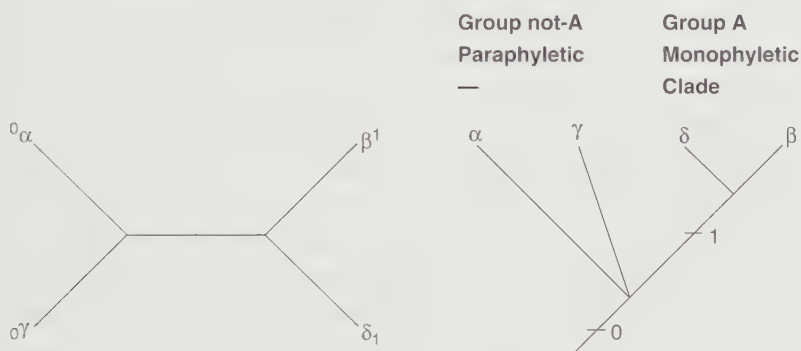


Figure 2 An unrooted tree for four taxa (left) and a rooted tree that can be derived from it (right). The unrooted tree shows the distribution among the four taxa (α – δ) of a single character with two states (0, 1), while the rooted tree (drawn with the root at the bottom) shows the evolutionary history of the character assuming that 0 is the ancestral state and 1 is the derived state. The rooted tree makes it clear that $\delta + \beta$ form an evolutionary group (group A) while α and γ do not form an evolutionary group and are merely the remaining taxa after recognizing Group A (group not-A). Thus, $\delta + \beta$ form a monophyletic group (all of the descendants of their most recent common ancestor) while $\alpha + \gamma$ form a paraphyletic group (only some of the descendants of their most recent common ancestor). Furthermore, the $\delta + \beta$ group can be referred to as a clade, while there is no equivalent term for α and γ because their grouping has no relevance in evolutionary history.

necessarily both of these partitions are evolutionary groups. It was Hennig (1966) who first made it clear that evolutionary groups can be recognized only by possession of shared derived character states in the taxa, as the ancestral character states were originally shared by all of the taxa. Thus, the rooted tree in Figure 2 makes it evident that only one of the two partitions in the unrooted tree is a true evolutionary group in this example—the other partition is merely the left-over taxa after we recognize the single evolutionary group. So, possession of derived character states among the taxa allows us to recognize monophyletic groups, while ancestral states only delineate paraphyletic groups. Monophyletic groups consist of all of the descendants of their most recent common ancestor (plus that ancestor), and thus they share unique characteristics in common due to their recent evolutionary history. This is of interest because it allows us to make predictions about unobserved commonalities, for example. Paraphyletic groups consist of only *some* of the descendants of their most recent common ancestor, and thus they do not necessarily share any particular characteristics at all—the characteristics that “define” a paraphyletic group were originally also shared with the remaining descendants of their most recent common ancestor, and thus are not unique to the paraphyletic group alone.

The bottom line here is that only monophyletic groups have an unambiguous evolutionary interpretation, and thus they are the only groups of interest that can be derived from a phylogenetic analysis. Such groups are called clades, and one of the most important objectives of most phylogenetic analyses is therefore the recognition of clades. For example, taxonomic groupings should always consist of clades, as should classifications of protein families, etc. So, each internal branch on an unrooted tree has a monophyletic group at one end and a paraphyletic group at the other, and we do not know which is which until we root the tree. (Note that if the root is on an internal branch then that branch will actually have a monophyletic group at each end.) Support for clades (monophyletic groups) can come only from a rooted tree—an unrooted tree can only be said to fail to reject a group, which is not the same thing as supporting that group.

Unfortunately, at the moment there is a common tendency for authors to try to interpret an unrooted tree by recognizing groups of

taxa, which they often then call “clusters” (or sometimes just “groups”). These consist of all of the taxa that are connected to a single internal branch on the tree. As I am emphasizing, these clusters will consist of both paraphyletic and monophyletic groups, depending on where the root is placed—if the root is outside the cluster then it is a monophyletic group, but if the root is inside the cluster then it is a paraphyletic group. Taxa that do not form a cluster constitute what is called a polyphyletic group. That is,

$$\text{clusters} = \text{monophyletic groups} + \text{paraphyletic groups}$$

but not polyphyletic groups. Thus, an unrooted tree allows us to recognize polyphyletic groups, but only a rooted tree allows us to distinguish monophyletic groups from paraphyletic groups. An unrooted tree is thus merely a stepping-stone, albeit an important one, to answering whatever biological question was originally posed. (As an aside, it is worth noting that the most obvious distinction between paraphyletic monophyletic groups and polyphyletic groups is that a polyphyletic group does not include the most recent common ancestor as part of the group, while the other two types of group do.)

I do not claim that interpreting relationships on an unrooted tree is completely unprofitable. As a step towards getting the final rooted tree, having an unrooted tree is obviously quite valuable. For example, as noted above, for nine taxa there are 135 135 unrooted binary trees each of which can be rooted in any one of 15 different places, yielding 2 027 025 possible rooted trees. Finding the unrooted tree thus eliminates 2 027 010 of these trees, leaving us with only 15 possible trees—this is clearly a major step, even if we never work out where the root actually is. Furthermore, an unrooted tree allows us to eliminate certain groupings from consideration (i.e. the polyphyletic groups) even though it does not allow us to recognize evolutionary groups (i.e. monophyletic groups)—that is, we can make negative statements about groups based on an unrooted tree, but we cannot make positive statements.

As a specific parasitological example, consider the unrooted tree shown in Figure 3. One of the questions asked by the original authors was whether their new microsatellite data were in concordance with the previously published isoenzyme or PCR–RFLP analyses of some

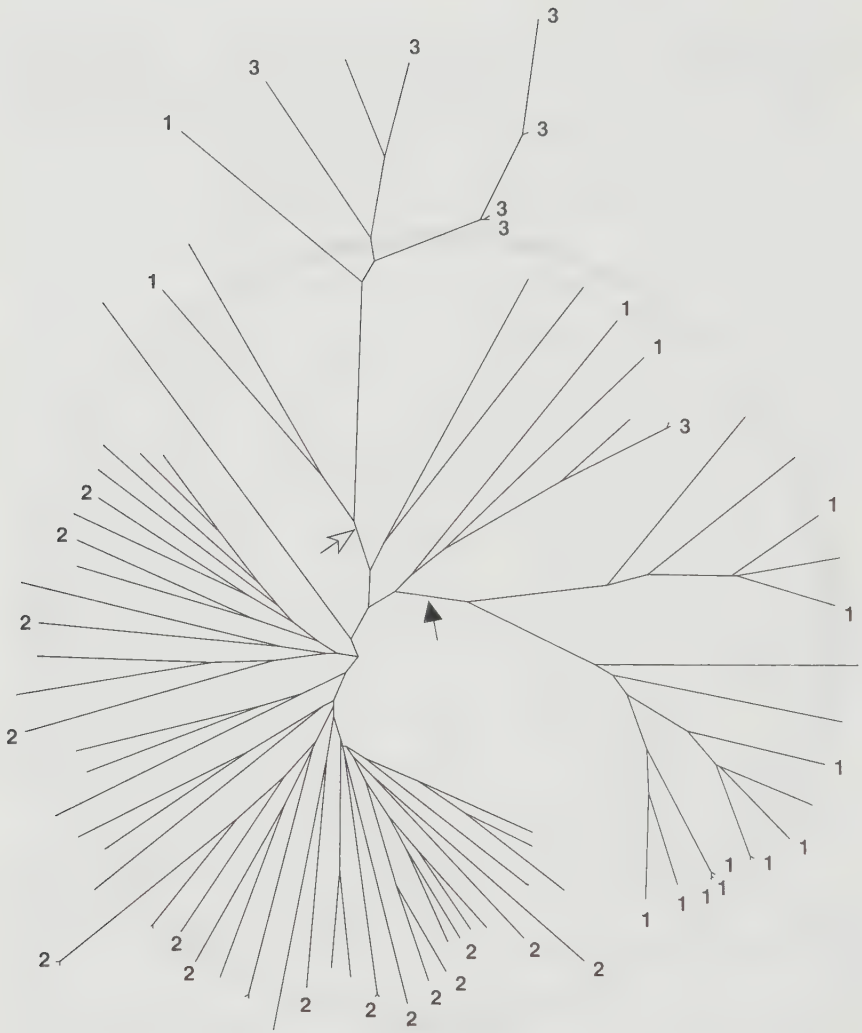


Figure 3 An unrooted tree for 83 samples of *Toxoplasma gondii*, based on microsatellite data. If the tree is rooted (in some manner) then this would represent a phylogenetic tree, with the branch lengths indicating the inferred amount of evolutionary change. The terminal labels refer to three zymodemes recognized for some of the samples based on prior isoenzyme or PCR RFLP analysis of those samples. The arrows refer to possible rooting positions, with the open arrow indicating the mid-point root and the filled arrow the molecular-clock root. The original data and analysis are from Ajzenberg *et al.* (2002).

of the same samples. This question can be answered solely with the unrooted tree, by comparing the zymodeme groups derived from the previous data with the clusters on the new tree. Zymodeme "2" forms a cluster on the tree, while zymodemes "1" and "3" do not. Therefore, zymodeme "2" is capable of forming a monophyletic group, while the other two zymodemes form polyphyletic groups. However, only by rooting the tree can we decide whether zymodeme "2" does actually form a monophyletic group or whether it forms a paraphyletic group instead.

3.2. Rooting Trees

This leads us on to the question of how best to root a phylogenetic tree. For molecular data, there are basically four ways that have been proposed (Huelsenbeck *et al.*, 2002a): (i) via reversible substitution models; (ii) mid-point rooting; (iii) using the molecular clock; and (iv) using an outgroup. Some of these methods have been more popular than the others, although not all of them are equally effective.

The first of these methods, (i), is based on the idea of imposing an evolutionary direction from within the tree-building analysis itself. This attempts to produce a rooted tree directly, rather than first producing an unrooted tree that is subsequently given a root (which is what the other three methods do). Most assessments of this method have revealed it to be relatively unsuccessful (Huelsenbeck *et al.*, 2002a), although there are exceptions (Yap and Speed, 2005), and so I will not discuss it further.

Mid-point rooting, (ii), refers to the simple choice of the point that is halfway along the longest pathlength between the terminal taxa. For example, the open arrow in Figure 3 is halfway between the pair of taxa at the top of the diagram and one of the taxa at the far right (which is a slightly longer pathlength than from the top to the bottom left), following along the branches of the tree. This idea is based on the principle that if the evolutionary rate has been constant among the taxa then all of the paths from the ancestor to the contemporary taxa should be of approximately equal length. This assumption may be reasonable, but its application clearly has one potentially serious

limitation: the calculation of the root location is based on only two of the taxa, and their evolutionary rate may or may not be representative of the other taxa. Figure 3 shows several taxa that have branch lengths that are longer than those of their immediate sisters, and these are the ones that will determine the position of the root.

The molecular clock, (iii), is an attempt to generalize the principle behind mid-point rooting. It takes into account all of the taxa simultaneously and roots the tree at what is effectively the average of the mid-points. This is indicated by the filled arrow in Figure 3, with the shaded circle shown as a heuristic device representing the "average" distance to the terminal taxa (i.e. the root is at the centre of the circle). Note that this root provides a different phylogenetic interpretation of the tree compared to the mid-point root. For example, zymodeme "1" forms a paraphyletic group based on the mid-point root, but a polyphyletic group based on the molecular-clock root. In the absence of any external information, the molecular-clock root is likely to be preferable to the mid-point root, although use of the latter has been a more popular method in the literature.

Use of an outgroup, (iv), is far and away the most widely used method of rooting a phylogenetic tree, and rightly so. The outgroup consists of one or (preferably) more taxa that are not part of the study group (i.e. the ingroup). The root of the tree is then simply the branch that connects the outgroup taxa to the ingroup taxa. This method thus uses information from outside the study group in order to identify the most likely placement of the most recent common ancestor (Smith, 1994). It is based on the principle that the outgroup should connect to the ingroup at the most recent common ancestor of the ingroup.

The main limitation of this method is the choice of the taxa to be included in the outgroup. Any specified set of outgroup taxa could be optimal for one ingroup, but suboptimal for another ingroup (e.g. when more taxa are added), and yet often the choice of outgroup apparently seems to be treated quite casually. However, its choice and analysis deserve detailed consideration in every phylogenetic analysis. Indeed, most phylogenetic controversies have at their heart only the rooting of the tree rather than any other aspect of the tree topology.

There are many difficulties associated with choosing an outgroup (Wheeler, 1990; Nixon and Carpenter, 1993; Smith, 1994; Barriel and

Tassy, 1998; Milinkovitch and Lyons-Weiler, 1998; Tarrío *et al.*, 2000; Holland *et al.*, 2003), not the least of which is that the evolutionary characteristics of the outgroup are likely to be quite different from those of the ingroup (almost by definition, because the outgroup and ingroup have had independent evolutionary histories since they diverged from their most recent common ancestor). Unfortunately, all of the various phylogenetic methods assume that the same evolutionary model applies across the whole tree, and if this assumption is violated then the resulting tree can be incorrect. The most likely place for this assumption to be violated is at the outgroup root, because the outgroup is by definition the evolutionarily most different part of the tree.

For robust phylogenetic analysis (Smith, 1994), the outgroup therefore needs to consist of several members of the sister taxon to the ingroup (i.e. the most closely related group to the ingroup), preferably the ones with relatively short branch lengths to the ingroup (i.e. the basal members of the sister group). Evolutionarily more-distant species can end up rooting the ingroup at what is effectively a random location, due to the lack of relevant phylogenetic signal involved in the long branch lengths leading to the outgroup. Alternatively, different rates of evolution in the ingroup and outgroup will also lead to long branches. When there are long branches homoplasy begins to play a large role, particularly parallelisms, which means that the long outgroup branches are more likely to attach to long ingroup branches than to short ones. Under these circumstances the outgroup serves no useful function, as the connection between the ingroup and outgroup is being determined by homoplasy rather than by homology.

Furthermore, many researchers seem to believe that their data are likely to contain a stable unrooted ingroup tree, and that adding an outgroup merely provides a root for that tree. However, this is not necessarily so. It has long been known that rooting a tree can change its topology (Neff, 1987), so that adding an outgroup to a data set can change the apparent ingroup relationships (Baum and Estabrook, 1996; Milinkovitch and Lyons-Weiler, 1998; Holland *et al.*, 2003), and that tree-building methods differ in the degree to which they are affected by this potential problem. This situation occurs because the tree-building methods are searching for global optimality and may

therefore sacrifice ingroup optimality if it is outweighed by optimizing the outgroup relationship. It can be particularly problematic if a part of the ingroup is quite different from the rest (i.e. a consistent evolutionary model does not apply even within the ingroup)—there is a tendency for the outgroup to join one of the ingroup taxa rather than to join on an internal branch of the ingroup tree, thus breaking up a clade within the ingroup (Baum and Estabrook, 1996). Analyzing the data both with and without the outgroup should reveal any such problems, and if the ingroup relationships change when the outgroup is added then it is likely that the tree without the outgroup is the more accurate one (Holland *et al.*, 2003). At worst, the ingroup topology may need to be estimated without the outgroup (i.e. the ingroup analyzed separately) and the outgroup added as a secondary step (Baum and Estabrook, 1996).

Note, also, that if you have not included all of the ingroup in your analysis then there will be some additional uncertainty associated with the root of the tree (i.e. the root node of the taxa you have sampled might not be the same as the root node of the complete ingroup). Under these circumstances, if you are going to identify the root of the tree correctly then you need to include in your sample of the ingroup at least one taxon from each of the two sister groups descended from the true root node (Sanderson, 1996). This might be done by trying to sample presumed basal taxa, or by sampling as widely as possible across the current taxonomic hierarchy (assuming that the taxonomy is related to some extent to the phylogeny).

3.3. Branch (Clade) Support

Since a phylogenetic tree is interpreted in terms of the monophyletic groups that it hypothesizes, it is important to quantitatively assess the robustness of all of these groups (i.e. the degree of support for each branch in the tree)—is the support for a particular group any better than would be expected from a random data set? This issue of clade robustness is the same as assessing branch support on the tree, since each internal branch on a rooted tree represents a clade. This directly addresses the subject of stochastic error in a phylogenetic analysis,

attempting to quantify the extent to which our sampling procedures have resulted in imprecise phylogenetic inferences. It is important to remember that a high degree of support cannot be interpreted as indicating a high degree of accuracy of the phylogenetic inferences, as the latter is related to systematic error instead. As noted in Section 2.1, accuracy depends on a complex set of characteristics many of which have nothing to do with branch support.

Most practitioners do not seem to realize it, but many different techniques have been developed to assess branch support, including: (i) analytical procedures, such as interior-branch tests (Nei *et al.*, 1985; Sneath, 1986), likelihood-ratio tests (Felsenstein, 1988; Huelssenbeck *et al.*, 1996c; Sanjuán and Wróbel, 2005) and clade significance (Lee, 2000); (ii) resampling procedures, such as the bootstrap (Felsenstein, 1985), the jackknife (Lanyon, 1985), topology-dependent permutation (Faith, 1991) and clade credibility (Larget and Simon, 1999); and (iii) non-statistical procedures, such as the decay index (Bremer, 1988), clade stability (Davis, 1993) and spectral signals (Hendy and Penny, 1993). Of these methods, far and away the most popular and widely used method has been the bootstrap technique (Soltis and Soltis, 2003), and so it is the one I will concentrate on in the next section.

However, first there is one general issue relating to branch support that needs to be discussed, in the sense that practitioners often seem to mis-interpret its importance. This issue appears to apply to all methods for assessing branch support not just to bootstrapping. This is the simple fact that branch support values cannot be independent of each other within a tree. For example, if a particular taxon is ambiguously placed in either of two different positions on a tree then there must be at least two branch-support values that are relatively small, one for each of the two placements (unless one of the placements is at the base of the tree). However, the situation is actually much worse than this because small branch-support values must also appear on *all* of the enclosing (i.e. more-inclusive) clades back to the most recent common ancestor of the two branches involved in the ambiguity (i.e. the least-inclusive common clade) – that is, low values will appear throughout the smallest monophyletic group containing all of the ambiguous placements, because clades are correlated.

for both placements. Consequently, the support for the clade Taxon 8 + Taxon 7 and for the clade Taxon 8 + Taxon 2 must each be 50%. However, the support for all other branches must also be 50% because every other branch on the tree also refers to a clade that might or might not include Taxon 8 (i.e. Taxon 8 is potentially a descendant of the ancestor represented by that branch), depending on where Taxon 8 is placed. So, in this example it looks superficially like the tree is not well supported at all. However, excluding Taxon 8 from the analysis (to produce what is called a pruned or reduced tree) reveals the true situation: all branches have 100% support, and thus the relationships among all of the other taxa are unambiguous. There is only one "problem" here but it creates five low bootstrap values.

This means that support values need to be interpreted carefully. A set of low support values on a tree may indicate uninformative data, or they may represent a small number of taxa with ambiguous placements. It is important to determine which situation you are dealing with (Lecointre *et al.*, 1993). Gatesy (2000) has highlighted a similar form of non-independence problem concerning decay index (bremer support) values, and the situation must exist for all other indices as well (e.g. Faith and Ballard, 1994).

3.4. The Bootstrap

This method was first introduced by Efron (1979) as an alternative method to jackknifing for producing standard errors on statistical estimates of central location other than the mean (e.g. the median), but it has since been expanded to cover probabilistic confidence intervals as well (Efron and Tibshirani, 1993; Davison and Hinkley, 1997). It was introduced into phylogenetic studies by Penny *et al.* (1982) and formalized by Felsenstein (1985), who suggested that it could be implemented by holding the taxa constant and resampling the characters randomly with replacement, the tree-building analysis then being applied to each of the bootstrap resamples. Since there is nothing particularly unique about the application of bootstrapping to phylogenetic trees, I will start by putting bootstrapping into a general context, as this makes the potential limitations clearer.

Bootstrapping is a Monte Carlo procedure that generates “pseudo” data sets from the original data, and uses these new data sets for its inferences. That is, it tries to derive the population inferences (i.e. the “true” answer) from repeated generation of new samples, each sample being constrained by the characteristics of the original data. It thus relies on an explicit analogy between the sample and the appropriate population: that sampling from the sample is the same as sampling from the population. Clearly, the strongest requirement for bootstrapping to work is that the sample be a reasonable representation of the population. Bootstrap confidence intervals are only ever approximate, especially for complex data structures, as they are a fundamentally more ambitious measure of accuracy than is a simple standard error for which they were originally designed. As a result, there are actually several quite distinct procedures for performing bootstrapping (Carpenter and Bithell, 2000), with varying degrees of expected success.

The original technique is called the percentile bootstrap. It is based on the principle of using the minimum number of *ad hoc* assumptions, and so it merely counts the percentage of bootstrap resamples that meet the specified criteria. The method is thus rather simplistic, and is often referred to as the naïve bootstrap, because it assumes no knowledge of how to calculate population estimates. It is a widely used method, as it can be applied even when the other bootstrap methods cannot be applied. However, it is known to have certain problems associated with the estimates produced, particularly for confidence intervals, such as bias and skewness (especially when the parent frequency distribution is not symmetrical), and thus it is actually the poorest form of bootstrap. This is the form of bootstrap introduced by Felsenstein (1985), and it is the one used by most phylogeny computer programs. It is therefore the one that will be discussed in more detail below.

These known problems with the naïve bootstrap can be overcome by using bias-corrected and accelerated (BCa) bootstrap estimates, so that the bias and skewness are both estimated and removed from the calculation of the confidence interval. The BCa method is thus the one usually recommended for use (Carpenter and Bithell, 2000). This method is much slower to calculate than is the simple percentile

bootstrap because it requires an extra parameter to be estimated for each of the bias and skewness corrections, and the latter correction is actually estimated by performing a separate jackknife analysis on each bootstrap resample (which means that the analysis can take 100 times as long as a naïve analysis). There have been several attempts to apply this form of correction methodology to bootstrapping in a phylogenetic context (Rodrigo, 1993; Zharkikh and Li, 1995; Efron *et al.*, 1996; Shimodaira, 2002), but while these can be successful at correcting bias and skewness (Sanderson and Wojciechowski, 2000) they have not caught on, possibly because of the time factor involved.

Alternatively, we can decide not to be naïve when calculating confidence intervals, and to calculate them in the traditional manner, using the standard error and the *t*-distribution. However, we then need to overcome any non-normal distributional problems of these two estimates by estimating both of them using bootstrapping. That is, bootstrapped-*t* confidence intervals are derived by calculating both the standard error (SE) and the *t*-value using bootstrapping, and then calculating the confidence interval as $\pm t^* \text{ SE}$. To many people, this is the most natural way to calculate confidence intervals, since it matches the usual parametric procedure, and thus it is frequently recommended (Carpenter and Bithell, 2000). Once again, this method is much slower to calculate than the percentile bootstrap, because the *t*-value is actually estimated by performing a separate bootstrap analysis on each bootstrap resample (which means that the analysis can take 100 times as long as a naïve analysis). This methodology seems not to have yet been suggested in a phylogenetic context, and in any case the time factor may be restrictive.

The above methods all count as non-parametric bootstrap methods. More recently, parametric bootstrapping methods have also been developed, which make the more restrictive assumption that a parametric model can be applied to the data (e.g. that the standard deviation of the parameter can be reliably estimated). In parametric bootstrapping, we generate simulated data sets based on the assumed frequency distribution of the data, rather than by resampling from the data set itself. That is, instead of sampling from the sample, we sample from the assumed theoretical distribution in order to generate the set of bootstrap samples. We can then apply the percentile, BCa

or bootstrap-*t* methods, as described above, in the usual way. Clearly, this method assumes that we know the appropriate frequency distribution, and the method will only be appropriate if this assumption is true. If this assumption is correct, then this can be the most powerful bootstrap method (Huelsenbeck *et al.*, 1996b; Newton, 1996) because it is not dependent on the representativeness of the data sample. The method has been introduced into phylogenetics in several contexts (Goldman, 1993; Adell and Dopazo, 1994; Huelsenbeck *et al.*, 1996b; Antezana, 2003), but the appropriate frequency distribution for branch support is not obvious (i.e. a phylogeny is a complex structure and cannot be represented by a single number but rather requires a model of sequence evolution and a model tree) and so it is not used for this purpose.

The point of this long explanation is to make it clear that, for several reasons, all of the best bootstrapping methods are not likely to be available when assessing the robustness of clades on a phylogenetic tree, and we are left with the naïve percentile bootstrap, which can be expected *a priori* to provide biased and skewed estimates of confidence intervals (because the frequency distribution associated with tree branches will not be symmetrical). Sadly, these problems have been repeatedly confirmed for the assessment of branch support in phylogenetic tree-building, both theoretically (Zharkikh and Li, 1992a, b; Felsenstein and Kishino, 1993; Li and Zharkikh, 1994; Sitnikova *et al.*, 1995; Berry and Gascuel, 1996; Efron *et al.*, 1996; Huelsenbeck *et al.*, 1996b; Newton, 1996; Sanderson and Wojciechowski, 2000; Suzuki *et al.*, 2002; Alfaro *et al.*, 2003; Erixon *et al.*, 2003; Goloboff *et al.*, 2003; Galtier, 2004; Yang and Rannala, 2005) and empirically (Sanderson, 1989; Hillis and Bull, 1993; Bremer *et al.*, 1999; Buckley *et al.*, 2001; Buckley and Cunningham, 2002; Wilcox *et al.*, 2002; Taylor and Piel, 2004).

These studies have demonstrated that the probability of bootstrap resampling supporting the true tree may be either under- or overestimated, depending on the particular situation. For example, bootstrap values $> 75\%$ tend to be underestimates of the amount of support, while they may be overestimates below this level. That is, when the branch support is strong (i.e. the clade is part of the true tree) there will be an underestimation and when the support is weak

(i.e. the clade is not part of the true tree) there will be an overestimation. This situation has been reported time and time again in the literature with various theoretical explanations (e.g. Felsenstein and Kishino, 1993; Efron *et al.*, 1996; Newton, 1996), although there are dissenting voices (e.g. Taylor and Piel, 2004). Unfortunately, practitioners seem to ignore this fact, and to assume that bootstrap values are always underestimates.

Just as importantly, the pattern of over- and underestimation depends on: the shape of the tree and the branch lengths, the number of taxa, the number of characters, the evolutionary model used and on the number of bootstrap resamples. For example, with few characters the bootstrap index tends to overestimate the support for a clade and to underestimate it for more characters. This is particularly true if the number of phylogenetically informative characters is increased or the number of non-independent characters is increased; and the index becomes progressively more conservative (i.e. lower values) as the number of taxa is increased. Moreover, these patterns of under- and overestimation are increased with an increasing number of bootstrap replications. These are serious issues, which seem often to be ignored by practitioners. We cannot just assume that the "true" support value is larger than our observed bootstrap value. In particular, this means that bootstrap values are not directly comparable between trees, even for the same taxa, and thus there can be no "agreed" level of bootstrap support that can be considered to be "statistically significant". A bootstrap value of 90% for a branch on one tree may actually represent *less* support than a bootstrap value of 85% on another tree, depending on the characteristics of the data set concerned and the bootstrapping procedure used (although within a single tree the values should be comparable).

This complex situation means that we have to consider carefully how best to interpret bootstrap values in a phylogenetic context (Sanderson, 1995). The bootstrap proportion (i.e. the proportion of resampled trees containing the branch clade of interest) has variously been interpreted as (Berry and Gascuel, 1996): (i) a measure of reliability, telling us what would be expected to happen if we repeated our experiment; (ii) a measure of accuracy, telling us about the probability of our experimental result being true; and (iii) a measure of

confidence, interpreted as a conditional probability similar to those in standard statistical hypothesis tests (i.e. measuring the rate of false positives). The bootstrap was originally designed for purpose (i), and all of the problems identified above relate to trying to use it for purposes (ii) and (iii). The values derived from the naïve bootstrap need correcting for purposes (ii) and (iii), and the degree of correction depends on the particular data set being examined (Efron *et al.*, 1996; Goloboff *et al.*, 2003).

There have also been attempts to overcome some of the practical limitations of bootstrapping for large data sets by adopting heuristic procedures, including resampling estimated likelihoods for maximum-likelihood analyses (Waddell *et al.*, 2002) and reduced tree-search effort for the bootstrap replicates. However, approaches using reduced tree-search effort produce even more conservative estimates of branch support, and the magnitude of the effect increases with decreasing bootstrap values (DeBry and Olmstead, 2000; Mort *et al.*, 2000; Sanderson and Wojciechowski, 2000).

3.5. Other Measures of Support

The discussion of these issues with the bootstrap leads inevitably to the consideration of other methods of measuring branch support, to see whether the same problems arise there as well. The problem of over- and underestimation seems to have been evaluated in the literature in any detail for only four other methods of branch support: interior-branch tests (Rzhetsky and Nei, 1992; although this is a test of branch length rather than branch support, see Yang, 1996b), topology-dependent permutation (T-PTP; Faith, 1991), jackknifing (Farris *et al.*, 1996), and clade credibility (Larget and Simon, 1999). For example, Huelsenbeck *et al.* (1996b) conclude that the a posteriori version of T-PTP almost always underestimates the true probability, while the a priori version may be slightly more accurate than the bootstrap value. Similarly, Mort *et al.* (2000), Goloboff *et al.* (2003) and Simmons *et al.* (2004) suggest that jackknifing underestimates support. Sitnikova *et al.* (1995) and Rzhetsky and Sitnikova (1996) suggest that the interior-branch test may give an overestimate

of statistical support, although this can be corrected to some extent, and is more accurate than the bootstrap.

Alternatively, clade credibility has been the subject of much recent investigation (Rannala and Yang, 1996; Wilcox *et al.*, 2002; Suzuki *et al.*, 2002; Alfaro *et al.*, 2003; Cummings *et al.*, 2003; Douady *et al.*, 2003; Erixon *et al.*, 2003; Simmons *et al.*, 2004; Zander, 2004; Mar *et al.*, 2005; Yang and Rannala, 2005). The consensus seems to be that clade credibilities cannot be used as a simple substitute for bootstrap values. For example, a clade credibility value is much less likely to be an underestimate of the true support than is a bootstrap value, and thus may be closer to the true probability. However, a large clade credibility value may actually be an overestimate of the true support, depending on the specific set of circumstances (e.g. the shape of the tree, the number of characters and taxa, the evolutionary model used, the specification of the prior probabilities). Thus, the situation seems to be just as complex as it is for bootstrapping, and it is not yet clear just how comparable clade credibility values are between trees, or whether there can be an "agreed" level of support that can be considered to be "statistically significant".

Perhaps the most interesting comparison of bootstrap and clade credibility values is the different assumptions to which they seem to be sensitive. Both of these methods are capable of providing strong support for incorrect branches on a tree, although they achieve this in somewhat different ways. Bootstrapping relies mostly on the quality of the original character sample and on the consistency of the tree-building method (Sanderson, 1995)—if the sample is biased then the resampling will be biased, and if the tree method is biased then the tree estimates will be biased in the resampling as well. Clade credibilities rely mostly on the specification of an adequate model of sequence evolution (Huelsenbeck *et al.*, 2002b; Huelsenbeck and Rannala, 2004) and appropriate priors (Yang and Rannala, 2005), because they explicitly represent updated probabilities based on the data in the light of the specified evolutionary model and the prior probabilities (see Section 6.2). They can therefore be sensitive to model mis-specification in the analysis (Huelsenbeck and Rannala, 2004; Lemmon and Moriarty, 2004; Nylander *et al.*, 2004), particularly under-parameterization (i.e. when a simple model is applied to complex data there can be strong

support for wrong groupings of taxa). For example, Suzuki *et al.* (2002) created a complex data set by combining several incompatible data partitions, and then applied a single model to the combined set. This simple model fits one of the partitions quite well, and so this partition gets strong support from the Bayesian analysis, contrary to the authors' expectations. In this case, the Bayesian analysis needs a different model for each partition (see Section 7.1) in order to produce the correct support values.

4. PHYLOGENETIC DATA

4.1. Taxon and Character Sampling

For a worthwhile phylogenetic analysis the data set needs to have complete or near-complete sequences, and reasonable taxon sampling. Taxon sampling and character sampling within the ingroup are therefore important issues for tree-building.

In general, more characters and more taxa are both better for phylogenetic analysis in the sense of increasing the accuracy of tree-building (Pollock *et al.*, 2002; Zwickl and Hillis, 2002), with character sampling becoming more important than taxon sampling once some minimal number of taxa have been sampled. The analysis of Goldman (1998) suggests that adding more characters (e.g. more genes) uniformly increases the phylogenetic information over the whole tree, while adding taxa increases the content only in some parts of the tree (i.e. where the taxon is placed). Moreover, Poe and Swofford (1999) have shown that the pattern of long and short branches on a tree, which is determined by the amount of taxon sampling, is what misleads most phylogenetic analyses, and this can be best dealt with by increasing character sampling rather than taxon sampling. Empirical studies comparing taxon and character sampling support these theoretical studies (Bremer *et al.*, 1999; Mitchell *et al.*, 2000; Simmons and Miya, 2004; DeBry, 2005).

For taxon sampling, what we need is a sample of replicate taxa from each of the clades within the ingroup (Lecointre *et al.*, 1993). If the sample is representative in the statistical sense, then using a subset

of taxa should not be a problem for phylogeny reconstruction (Rosenberg and Kumar, 2001; Rokas and Carroll, 2005), while if it is not representative then there will be inconsistencies among trees derived from different samples of taxa (Rydin and Källersjö, 2002). In particular, it is important to sample the basal members of the phylogenetic tree, as this will ensure that the root of the tree is determined as accurately as possible and that the full "width" and "height" of the phylogenetic tree are determined. In the absence of any evidence to the contrary, the current taxonomy of the organisms is probably the best framework to use for deciding which groups of taxa are likely to form monophyletic groups or to be basal, and thus should be sampled. This taxonomy may not be phylogenetic, but it will be biologically based and thus will be better than no framework at all.

Just how many taxa are needed, however, is not clear. For example, many of the theoretical studies to date have been based on random sampling of taxa, which is clearly not how biologists behave (see below), and thus may be of little relevance—expectations of standard sampling theory do not necessarily apply in phylogenetics. If the entire ingroup is not sampled, then in general more taxa may be better than fewer (Poe, 1998a) because model estimation is more accurate under these circumstances (Pollock and Bruno, 2000). However, the percentage of taxa sampled from each clade is probably more important than the actual number sampled (Yang and Goldman, 1997; Poe, 1998b; Rannala *et al.*, 1998), because an increased percentage decreases the average length of the external branches of the tree, which increases the accuracy of tree-building. Furthermore, the taxa need to be sampled in such a manner as to avoid juxtaposed short and long branches on the resulting tree if possible (Graybeal, 1998; Lyons-Weiler and Takahashi, 1999)—these will be "informative taxa" that increase accuracy rather than "redundant taxa" that might decrease it. If taxon sampling occurs at random, then on average increased sampling will decrease the lengths of external branches, which increases accuracy (Rannala *et al.*, 1998).

It has been shown consistently in the literature that poor taxon sampling is usually the result of biased selection of taxa (Swofford *et al.*, 1996). Most experimenters purposively choose their taxa, and this can be advantageous because taxa can be chosen for their potential

value in resolving the phylogeny (Hillis, 1998)—if exemplar taxa are chosen carefully then sampling can be beneficial because it reduces systematic error. Unfortunately, in parasitology taxon sampling is usually opportunistic (Barta, 2001), which clearly cannot create either a statistically representative sample or a purposive sample (a “patchwork” is probably a better description). For example, early studies of the *Coccidia* often came to what is now considered to be a wrong conclusion, or the right conclusion for the wrong reason, because so few species were included in the sample, which then showed supposedly “close” relationships among taxa solely because more closely related species were missing from the analysis (Holmdahl *et al.*, 1999). A similar conclusion was reached by Hughes and Piontkivska (2003) regarding the Trypanosomatidae, where poor sampling of taxa for a range of protein families severely limited the usefulness of the analyses.

If multiple sequences are available for a particular taxon then merging the replicate sequences into a single consensus sequence can be a helpful strategy for a phylogenetic analysis, because it takes into account intra-species genotypic variation. More usually, for taxa with multiple sequences one sequence is chosen as “representative” and the rest are discarded. However, I cannot recommend this latter strategy because then the phylogeny actually represents the history of a set of individuals rather than the history of a set of species. The alternative strategy, of course, is to include all of the duplicate sequences as separate taxa in the data analysis (e.g. Lopez *et al.*, 1999; Šlapeta *et al.*, 2002). This will be especially helpful if the sequences represent different gene loci, such as are produced by gene duplication, which can then have different evolutionary histories (unless concerted evolution has occurred). Expression of different gene loci in different developmental stages is not uncommon in some types of parasites, especially those with multi-host life cycles (e.g. Rooney, 2004), and this variation may be important for parasitologists.

As far as character sampling in a phylogenetic analysis is concerned, there are three main issues: which characters to choose, how many to choose, and what to do about missing data. The first two issues are related, of course, and for molecular data it may sound like a good idea to sequence whole genomes because it reduces random error. Enough mitochondrial genomes are becoming available

for useful phylogenetic analyses (e.g. Lin *et al.*, 2002; Mu *et al.*, 2005) but not enough nuclear genomes are (e.g. Rokas *et al.*, 2003), and thus this approach is currently inadequate. In addition to this is the problem of faulty assembly of the genomes, a common but often-overlooked problem (Salzberg and Yorke, 2005), which will have serious effects on phylogenetic analysis. So, in the near future we will need to continue to make a choice of characters.

In making a choice it is important to recognize that it is not the number of characters *per se* that is important but rather the number of phylogenetically informative characters. Characters that are invariant or character states that are restricted to a single taxon are not informative about the branching order of the phylogeny, although they may be informative about the branch lengths. So, considerations such as gene length or number of microsatellites or number of restriction sites are only important if increases lead directly to increases in the number of informative characters. There have been several empirical studies, for example, showing that gene length and success in reconstructing a tree can be unrelated (e.g. Steinbachs *et al.*, 2001), although there will be some minimum number of characters below which it is impossible to discriminate between alternative phylogenetic trees.

The choice of the number of informative characters will be determined mainly by how many short internal branches there are on the tree, although the distribution of short terminal branches also has an effect. Short internal branches are the hardest for the tree-building analyses to detect and the hardest for which to get good length estimates. So, you need enough characters to estimate all of these branch lengths accurately, and the number of such branches is related to the number of taxa. Those studies done (Churchill *et al.*, 1992; Lecointre *et al.*, 1994; Cummings *et al.*, 1995; Poe and Swoford, 1999; Dopazo *et al.*, 2004; Delsuc *et al.*, 2005; Wortley *et al.*, 2005) suggest that, from a statistical perspective, the number required for 95% confidence in branch lengths can be depressingly large (in the tens or hundreds of thousands; although cf. Hillis, 1996), because there is decreasing return for effort as the number of characters is increased. However, the number of characters required increases less than linearly with the number of taxa (Bininda-Emonds *et al.*, 2001).

Adding fewer characters for all taxa is better than adding many characters for only some of the taxa (Wiens, 1998).

As for which characters to choose, the trivial answer is: those that will accurately represent the phylogeny of the taxa. Given that we do not know a priori which these are (although it is often suggested that mitochondrial or chloroplast sequence data are a good bet, because they are usually maternally inherited with little recombination: Rubinoff and Holland, 2005), instead we choose those with enough variability to provide a phylogenetic signal but not so much that the signal has been lost. For example, starting from identical sequences, an increase in sequence divergence among the taxa rapidly increases the accuracy of tree-building as phylogenetic signal is added, followed by a slow decline as multiple substitutions begin to obscure the signal (Yang, 1998). Beyond about 30–40% sequence divergence the number of multiple substitutions can become saturated, and serious loss of phylogenetic signal will ensue. This means that for closely related taxa you will need genes with a high rate of evolution, while for more distantly related taxa you will need more-conserved genes (the rate of evolution is determined by the functional constraints, so quickly evolving genes have fewer constraints). This, in turn, implies that you will probably need several different genes in the same analysis, apart from the general principle that a tree from a single gene is unlikely to represent the true species phylogeny (Cummings *et al.*, 1995). Certainly, at low levels of sequence divergence adding more sequence length can have a dramatic effect.

When dealing with sequence data it is common for there to be missing characters, either because some taxa could not be completely sequenced for a particular gene or sometimes could not be sequenced at all for that gene. Characters with missing data should be excluded from the analysis if they result from technical problems that can cause ambiguity, such as sequence repeats or inadequate priming. However, including incomplete taxa in a tree-building analysis is otherwise more likely to increase the accuracy of the tree-building than to decrease it (Wiens, 1998, 2005; Wiens *et al.*, 2005), although the degree depends on the proportion of missing data. Taxa with lots of missing data are more problematic than are characters with lots of missing data, as these can be positively misleading (Wilkinson, 1995b; Wiens, 1998).

Another issue worth mentioning is that the characters themselves need to match the assumptions of the evolutionary model that will be used for their analysis. The most important of these assumptions is stationarity (see Section 5.1), so that stationary genes are to be preferred to non-stationary ones (Collins *et al.*, 2005). Moreover, the sorts of complex evolutionary models discussed in Section 5 can require large numbers of nucleotides and large numbers of taxa for accurate estimation of their parameters (Nei *et al.*, 1998; Sullivan *et al.*, 1999; Sullivan and Swofford, 2001; Blouin *et al.*, 2004), and not all tree-building methods will necessarily be able to find the phylogenetic signal in any particular molecular data set (Steinbachs *et al.*, 2001). This thus constitutes another reason to carefully consider taxon and character sampling. So does the estimation of character states for the inferred ancestors, which I argued in Section 3.1 is often of interest in addition to tree topology. Increased taxon sampling is beneficial as it increases the information available for estimating the character states at the root of the tree (Salisbury and Kim, 2001).

Also, adding characters to a data matrix can actually make the tree-building analysis run faster (Hillis, 1996; Soltis *et al.*, 1998). This is because the extra characters are unlikely to contradict all of those already in the data set (assuming that they are mostly informative about the phylogeny) but will instead increase the signal-to-noise ratio. This, in turn, will reduce the number of trees that get examined during the analysis, which will more than counterbalance any increase in calculation time due to the extra characters (which may be small anyway, because characters that show exactly the same pattern across the taxa are usually automatically combined for analysis). Finally, it should be obvious that taxon sampling affects automated sequence alignment methods, since they search for globally optimal solutions and will thus respond to the particular context of sequences being aligned.

It is perhaps worth also noting that the modern “post-genomic” era has (temporarily, I hope) regressed to the Bad Old Days, in which inadequate taxon sampling is overlooked in the excitement generated by large amounts of new characters (Delsuc *et al.*, 2005). This means that papers are now being published that claim to be testing evolutionary (and taxonomic) hypotheses using whole-genome sequences.

but without the remotest hope of adequately testing the stated hypotheses because the taxon sampling is wholly insufficient. For example, Dopazo *et al.* (2004) claimed to perform a test of two competing taxonomies of part of the Metazoa: the group Ecdysozoa (moulting animals, including nematodes and arthropods) versus the groups Pseudocoelomata (nematodes) and Protostomata (arthropods, annelids, molluscs). However, they attempted to do this with two insects but no arachnids or crustaceans (which form the rest of the Arthropoda), a single nematode, and without any molluscs or annelids at all. Clearly, the results of such a test are worthless, no matter how many characters are involved (25 676 nucleotide positions in this case). Most of the genome sequences currently available are for the so-called model organisms, and these single species are neither representative of the taxonomic groups from which they come nor do they cover more than a small fraction of the relevant taxonomic groups. More to the point, replication is a basic tenet of science, and without replicating taxa from each of the relevant groups it is difficult to make the claim that such analyses are a part of high-quality science.

4.2. Data Deletion

When dealing with morphological or anatomical data it is usual to decide *a priori* which characters will be sampled and which ones will not. However, when collecting molecular data this only applies to the choice of genes to be sequenced, or to the primers to be used (e.g. for RAPD, AFLP), etc. It does not apply to the actual data collected. This leaves the experimenter open to choose to include or exclude the observations at will *after* the data have been collected. For example, it is quite common when dealing with DNA sequence data to exclude characters that cannot be aligned unambiguously, alignment positions that appear to be overly variable or saturated (such as 3rd-codon positions), or even simply positions where gaps have been introduced into the alignment. A cautionary note is therefore warranted.

The general argument for deleting these sorts of regions is that they are more likely to contain homoplasy than are the other regions. This

is true but irrelevant. Homoplasy can be just as informative about phylogenetic relationships as is homology. That is, parallelism, convergence and reversal can all be real evolutionary events, and therefore detecting them can be effective for reconstructing evolutionary history. Homoplasy on a phylogenetic tree can therefore reveal true relationships among the taxa. The only exceptions to this will be homoplasies that result from wrong data or an incorrectly specified alignment or tree-building analysis. In particular, it seems quite likely that molecular characters will be homoplasious in some parts of a tree and not others, due to varying evolutionary rates among taxa (Philippe *et al.*, 1996). Therefore, in general, excluding (or down-weighting) characters due to homoplasy can be counter-productive for the robust reconstruction of phylogeny. This creates a potential conflict, of course—homoplasy can be both “good” and “bad” for a phylogenetic analysis, depending on the particular characteristics of the data set (see Section 2.2), and so the consequences of homoplasy require careful thought on the part of the practitioner.

Deleting variable sequence regions, in particular, can be unwise (Lutzoni *et al.*, 2000; Lee, 2001). For instance, the alignment ambiguity might result from only one taxon, or affect only one taxon, with the other sequences being readily alignable in that region (Lee, 2001). Alternatively, for ribosomal DNA, for instance, alignment-ambiguous regions will usually be those parts of the sequence representing single-stranded loops between the helices, and there is no a priori reason to expect that phylogenetic signal will be absent from these regions (Morrison and Ellis, 1997; Beebe *et al.*, 2000; Mugridge *et al.*, 2000). In other words, the phylogenetic signal in the variable regions can supplement that in the regions of unambiguous alignment, but it may or may not be congruent with it. For example, Morrison *et al.* (2004) reported that in their analyses of the small subunit rRNA of the Sporozoa there were clear shared derived character states within these regions, and these synapomorphies accounted for some of the differences in support for the taxonomic groups compared to those found by previous researchers. For example, the region consisting of the E21-1, E21-3 and E21-5 helices was a region of relatively unambiguous alignment for the Eimeriidae and Toxoplasmatinae but was very variable for the Sarcocystinae. Deleting this region from any

phylogenetic analysis would therefore reduce the amount of apparent support for the distinction of these taxonomic groups.

Furthermore, the phenomenon that gapped regions of a sequence alignment can contain valuable phylogenetic information has been noted by many authors (Lloyd and Calder, 1991; Söltmann *et al.*, 1995; Cerchio and Tucker, 1998; Giribet and Wheeler, 1999; van Dijk *et al.*, 1999; Graham *et al.*, 2000; Freudenstein and Chase, 2001; Poux *et al.*, 2002; de Jong *et al.*, 2003; Kawakita *et al.*, 2003). From this perspective a gap inserted into a sequence alignment represents an indel, which can be thought of as a real biological event (see Section 5.3)—although we can never observe whether it was an insertion or a deletion, we can often infer which it was once we have a phylogenetic tree. Thus, an inferred indel may contain the same sort of phylogenetic information as an inferred substitution. The incorporation of gapped regions into a phylogenetic analysis is simply part of the general search for phylogenetic signal in whatever genomic changes have occurred in the sequences (Rokas and Holland, 2000; Simmons and Ochoterena, 2000; Simmons *et al.*, 2001). Similar arguments have been mounted for 3rd-codon positions in protein-coding genes, which are often excluded on the grounds that they are too variable and may therefore represent homoplasy, but which may actually contain most of the phylogenetic signal (Cummings *et al.*, 1995; Yang, 1996b, 1998; Håstad and Björklund, 1998; Björklund, 1999; Källersjö *et al.*, 1999; Wenzel and Siddall, 1999), since homoplasy can represent true phylogenetic information as well as error.

So, although there are objective criteria for deleting regions of variable or ambiguous alignment in phylogenetic analyses (Castresana, 2000), there are alternative strategies for dealing with the difficulties that inevitably arise if you try to keep them in your analysis. Three of these are reviewed by Lee (2001), which he calls multiple analysis (Lee, 2001), elision (Wheeler *et al.*, 1995) and fragment-level analysis (Wheeler, 1999; Lutzoni *et al.*, 2000). In addition, there is the staggered alignment method of Barta (1997). These methods are all preferable to simply discarding characters by deleting variable, ambiguous or gapped parts of an alignment.

All this having been said, it is important to remember that regions with indels are subject to the same processes of homoplasy (e.g.

convergence and reversal) as are other sequence regions. This means that apparent phylogenetic signals in these regions can be just as misleading as they can elsewhere (Ford *et al.*, 1995; Baptiste and Philippe, 2002; Krzywinski and Besansky, 2002), and so caution is still required when deciding which sequence regions to include in any phylogenetic analysis. Unfortunately, we are currently only just starting to develop explicit methods for incorporating the information from gaps into likelihood models (see Section 5.3), such as there is for the simpler parsimony methods (Wheeler *et al.*, 1995; Wheeler, 1999; Lutzoni *et al.*, 2000; Simmons and Ochoterena, 2000; Lee, 2001). It is likely, however, to be possible to include indels as a fifth character state (McGuire *et al.*, 2001) or to code gaps as a separate set of binary characters (Young and Healy, 2003).

5. EVOLUTIONARY MODELS

All data analyses are based on some form of underlying model, whether explicit or implicit, which specifies the assumptions that need to be met by the data in order for the results of the analyses to be reliable (Penny *et al.*, 1994). In a phylogenetic analysis of sequence data, the model most commonly takes the form of assumptions about the process of nucleotide (or amino acid) substitution that will be used to infer the unknown events of the evolutionary history, based on the data concerning the distribution of nucleotide (or amino acid) frequencies among the contemporary sequences (Steel and Penny, 2000). The current trend is to explicitly use such models in the tree-building analysis, and to develop increasingly more sophisticated models. I will therefore review the current state of play in some detail and indicate possible future directions.

Evolutionary events happen to the DNA (for most organisms), and so the DNA models are based on attempts to model the biological processes that determine these events. Amino acid models, on the other hand, are currently mostly empirical, in the sense that the substitution probabilities are based on observations of real sequences rather than on any theoretical considerations. For both cases, the models that have been developed are quite general, in the sense that they describe

evolutionary processes by specifying only a set of basic parameters. These parameters specify the rates at which different evolutionary processes occur. However, the user does not need to know the actual values of the parameters, because these are estimated during the tree-building analysis.

The choice among phylogenetic models should be quantitatively assessed rather than arbitrarily chosen (Johnson and Omland, 2004). Choosing to compare the results of a range of phylogenetic methods without explicit justification for their choice is a very rough heuristic analysis indeed, because it does not assess the suitability of the models underlying each of the methods for the data at hand. For example, knowing which groups of taxa are well supported by an inappropriate evolutionary model is not particularly useful—it is far better to know something about the adequacy of the models. If there is less than about 90% identity among the sequences then we should suspect the potential existence of problems with our models (Rzhetsky and Sitnikova, 1996). Consequently, the adequacy of the models needs to be tested: if a too-simple model is used then it will not reflect reality, which can lead to biased inferences (e.g. usually underestimates of the true parameter values, especially branch lengths), and if a too-complex model is used then it will be over-parameterized, which leads to inflated variances (i.e. less precision of the parameter values) and unnecessary computational complexity (Yang, 1996b; Håstad and Björklund, 1998; Sullivan *et al.*, 1999; Posada, 2001). What is needed is a model that is an adequate description of the data and no more. It is beside the point how “realistic” the model is, since all models are definitely wrong by being over-simplifications—after all, Isaac Newton developed his successful theory of gravity by assuming that all planets can be treated as infinitesimal points, which we all suspect to be an unrealistic over-simplification of the true situation. Lack of realism does not matter provided the model is robust (Sullivan and Swofford, 2001).

Note that model complexity on its own is not necessarily a good thing, as it always comes at a cost (Steel, 2005). For example, there is an increasing computational burden with increasing complexity, although this affects different tree-building methods to different degrees. Furthermore, it is not unknown for simpler and obviously

incorrect models to yield more accurate answers than do more complex models (Yang, 1997a; Posada and Crandall, 2001b; Steinbachs *et al.*, 2001) –that is, the best-fitting model does not necessarily lead to the correct phylogenetic tree (Ren *et al.*, 2005). This may occur for a number of reasons, including correlations among branch parameters (Lyons-Weiler and Takahashi, 1999), fortuitous biases inherent in the simpler model (Bruno and Halpern, 1999), and over-correction for multiple substitutions when sequence lengths are relatively short (Holland *et al.*, 2003). Basically, estimation in a phylogenetic context is not a straightforward statistical exercise, because each tree has its own parameter space and a different probability function (Yang *et al.*, 1995).

For sequence data it is worthwhile distinguishing three evolutionary models, all of which are involved in a phylogenetic analysis: the substitution model, the indel model, and the tree model. Simplistically, the substitution model deals with multiple substitutions of nucleotides or amino acids, the indel model deals with unequal sequence lengths, and the tree model deals with branch lengths. There is more to it than this, of course, but the conceptual distinction between the three models has important practical consequences for how phylogenetic analyses are carried out, as discussed next.

5.1. Nucleotide Models

For DNA sequence data, the substitution models work by estimating the expected amount of nucleotide divergence between the sequences, assuming some specified probability that nucleotide changes will occur during any specified evolutionary time-period. They are based on what is mathematically described as a time-continuous homogeneous Markov process, which then provides a set of rates at which each nucleotide is substituted by each alternative nucleotide per nucleotide site per unit of evolutionary time. The parameters that affect these rates include the relative frequencies of the bases, the rates of change among bases through time, the ability of bases at different alignment positions to change, and whether the rates of change remain constant through time (see Figure 5). It is possible to fix any of these model

	Base frequencies $P_A = P_C = P_G = P_T$	Nucleotide substitutions $A \leftrightarrow C = A \leftrightarrow G = A \leftrightarrow T =$ $C \leftrightarrow G = C \leftrightarrow T = G \leftrightarrow T$	Among-site rate variation $I = 0; \alpha = \infty$
Stationary models Tests available:	(a) (i) likelihood-ratio test (ii) AIC (iii) decision theory	(b) likelihood-ratio tests / AIC / decision theory for combinations of ti and tv	(c) likelihood-ratio tests / AIC / decision theory for (i) $\alpha = \infty$; (ii) $I = 0$
Non-stationary models Tests available:	(d) (i) overall/individual χ^2 (ii) matched-pairs test (iii) disparity index	(e) (i) symmetry χ^2 (ii) triplet markov (iii) heuristic χ^2	(f) (i) Huelsenbeck test (ii) Lockhart–Steel test

Figure 5 Conceptual scheme for specifying a nucleotide substitution model in the phylogenetic analysis of molecular sequences. There are three basic components to any evolutionary model, indicated by the three columns. Explicit assumptions are made in the model as to whether each component is constant or not: whether the base frequencies are assumed to be all equal (column 1), whether all nucleotide substitutions are assumed to occur at the same rate (column 2), or whether all site positions in the sequence are assumed to have the same rate of substitution (column 3). Furthermore, explicit assumptions are made about whether each component of the model is constant across the evolutionary tree (referred to as a stationary model, row 1) or whether the model component can vary among different lineages (referred to as a non-stationary model, row 2). There are thus six different aspects of an evolutionary model that can potentially affect the results of a phylogenetic analysis, labelled (a)–(f) in the figure, and which should be tested to determine the most appropriate assumptions for the data at hand. For example, the most basic model assumes that all six aspects are constant, and this is the Jukes–Cantor model. On the other hand, if (a) + (b) + (c) are assumed to be variable but (d) + (e) + (f) are constant then this is the GTR + I + Γ model, which is the most sophisticated model available in most phylogeny computer programs. P_A – P_T are the relative proportions of the four bases; $A \leftrightarrow C$ – $G \leftrightarrow T$ are the relative rates of the six patterns of possible nucleotide substitutions; I is the proportion of aligned sequence sites assumed to be invariable; and α is the shape parameter of the gamma distribution that is used to describe the variation in substitution rate among the variable sites. Various possible statistical tests are listed for each model aspect, as discussed in the text.

parameters at specified values (perhaps by assuming that they are all equal across the sequences or equal through time) or to estimate them from the data (perhaps by the mathematical procedure of likelihood).

These models can be used to provide “corrected” distances when using distance-based tree-building methods, and they will then provide better estimates than using raw dissimilarity measures between sequences. However, these models come into their own when using likelihood-based techniques, such as maximum likelihood or Bayesian analysis (see Section 6). The use of likelihood models has become increasingly prevalent in tree-building analyses (Lewis, 2001b; Holder and Lewis, 2003), and so they are worth exploring in more detail here. Although I am concentrating mainly on sequence-based models, similar principles can also be applied to morphological data (Lewis, 2001a).

The concept that relatively simple molecular models could be developed was first suggested by Jukes and Cantor (1969), who developed a probability model that allows correction for multiple substitutions of nucleotides through time. Since then, suggestions have been made that allow the models used for nucleotide data to incorporate possible variation in successively more complex characteristics (Figure 5): nucleotide substitution (Kimura, 1980), nucleotide composition (Felsenstein, 1981), and among-site rate variation (Jin and Nei, 1990; Yang, 1993). Thus, the most complex model currently available allows the base frequencies to vary, all six possible substitution rates to vary (GTR; Tavaré, 1986), a proportion of the sites to be invariant (I; Hasegawa *et al.*, 1985) and the variable sites to vary along the sequence according to a discrete gamma distribution (Γ ; Yang, 1994). This leads to the GTR + I + Γ model (Gu *et al.*, 1995). The models that are in common use are listed in Table 1 in order of increasing complexity.

In my experience, far and away the biggest effects created by the parameters of the model relate to the treatment of positional variability, both with respect to assuming that some proportion of the sites are invariable (Steel *et al.*, 2000b) and to how much variability there is among those sites that do vary (Yang, 1996a). This has been commonly observed by other people (e.g. Buckley *et al.*, 2001; Sullivan and Swofford, 2001; Buckley and Cunningham, 2002; Lemmon and Moriarty, 2004; Nylander *et al.*, 2004) as well. It presumably has to do with multiple substitutions, as severe rate variation among sites means that there are relatively few sites containing phylogenetic information.

Table 1 The 56 possible single-nucleotide models that are currently available for phylogenetic analysis via likelihood

Model ^a	Reference	Base frequencies	Nucleotide substitutions ^b	No. free parameters ^c
JC	Jukes and Cantor (1969)	Equal	Equal	0
F81	Felsenstein (1981)	Unequal	Equal	3
K80	Kimura (1980)	Equal	Ti≠Tv	1
HKY	Hasegawa <i>et al.</i> (1985)	Unequal	Ti≠Tv	4
TrNef		Equal	Ti ₁ ≠Ti ₂ ≠Tv	2
TrN	Tamura and Nei (1993)	Unequal	Ti ₁ ≠Ti ₂ ≠Tv	5
K81(K3P)	Kimura (1981)	Equal	Ti≠Tv ₁ ≠Tv ₂	2
K81uf		Unequal	Ti≠Tv ₁ ≠Tv ₂	5
TMef		Equal	Ti ₁ ≠Ti ₂ ≠Tv ₁ ≠Tv ₂	3
TIM		Unequal	Ti ₁ ≠Ti ₂ ≠Tv ₁ ≠Tv ₂	6
TVMef		Equal	Ti≠Tv ₁ ≠Tv ₂ ≠Tv ₃ ≠Tv ₄	4
TVM		Unequal	Ti≠Tv ₁ ≠Tv ₂ ≠Tv ₃ ≠Tv ₄	7
SYM	Zharkikh (1994)	Equal	Ti ₁ ≠Ti ₂ ≠Tv ₁ ≠Tv ₂ ≠Tv ₃ ≠Tv ₄	5
GTR(REV)	Rodríguez <i>et al.</i> (1990)	Unequal	Ti ₁ ≠Ti ₂ ≠Tv ₁ ≠Tv ₂ ≠Tv ₃ ≠Tv ₄	8

^aEach listed model comes in four variants: standard, +I, +Γ, +I+Γ. I = proportion of aligned sequence sites are assumed to be invariable; Γ = gamma distribution is used to describe the variation in substitution rate among the variable sites.

^bThere are two possible transitions (Ti) and four possible transversions (Tv), with the transversions potentially grouped into two pairs.

^cThe number of parameters needing to be estimated in the analysis. There is one additional parameter for each model that includes I and one additional parameter for each model that includes Γ. There are also 2*n* - 3 branch-length parameters in the tree model.

and multiple substitutions at these few sites will rapidly obscure the phylogenetic signal (Yang, 1998). Therefore, the use in phylogenetic analyses of models that do not take this source of variation into account may be rather inadequate.

All of the models discussed so far make the same basic assumption that the model does not change through time along the evolutionary lineages (or, if you prefer, in different subtrees). That is, sequence divergence is good for tree-building, because it is the patterns of sequence difference among taxa that are being analyzed, but divergence

among taxa needs to be of the same type over the whole tree – this will be the case if evolution is assumed to be neutral. If this is so, then mathematically the model is said to be stationary. Non-stationarity (i.e. deviation from the standard model) creates analogy rather than homology among the characters. Analogies created by character-state reversals are dealt with in the models by correcting for multiple substitutions (although multiple substitutions will be underestimated in stationary models if there is non-stationarity), but analogies created by parallelism and convergence are harder to deal with. Note that almost all of the potential “problems” that have been highlighted in phylogeny reconstruction over the years, such as long-branch attraction and compositional bias, are merely specific examples of non-stationarity and therefore of homoplasy, and so they are not actually different problems but are merely separate manifestations of the same problem. Basically, if there is homoplasy in the data (caused by any molecular mechanism that you can think of) then we may expect the possibility that wrong branches will join on the tree.

Biologically, stationarity is an unlikely assumption, because the physical constraints on the macromolecule coded for by the gene are likely to have varied through time, and so the DNA sequence is expected to have been subjected to temporal variation as well (Penny *et al.*, 2001). Therefore, we need to deal with non-stationarity in some effective manner, and suggestions have recently been made that allow for temporal variation in parameters of likelihood models such as nucleotide composition (Yang and Roberts, 1995; Galtier and Gouy, 1998; Foster, 2004), transition:transversion ratios (Yang and Yoder, 1999), evolutionary rates (Bickel and West, 1998; Cutler, 2000; Huelsenbeck *et al.*, 2000) and among-site rate variation (Tuffley and Steel, 1998b; Galtier, 2001; Huelsenbeck *et al.*, 2002b). Unfortunately, few of these suggestions have yet been incorporated into the most commonly used computer programs, mainly because they do not fit easily as extensions of the current simpler models; and there are very few assessments of the effects on non-stationarity on phylogenetic inference (Ho and Jermini, 2004).

However, the covarion covariotide model has come to prominence in a number of recent phylogenetics publications (Lockhart *et al.*, 1998; Tuffley and Steel, 1998b; Penny *et al.*, 2001; Galtier, 2001;

Huelsenbeck, 2002; Lopez *et al.*, 2002; Morrison *et al.*, 2004), even though it actually has a 35-year history (Fitch and Markowitz, 1970). In this model, the rates of nucleotide (or amino acid) substitution are allowed to vary between evolutionary lineages—that is, as an addition to the GTR + I + Γ model, which allows substitution rates to vary at different alignment positions, the substitution rate at any position is allowed to be different in different taxon groups or subtrees (= lineages on the tree). Basically, the model allows each site to alternate between “on” and “off” as far as substitutions are concerned, so that there is a change along the branches of the tree as to which sites are the variable ones, even if the proportion of variable sites remains constant throughout the tree. This model can potentially change the shape of the tree, and will almost certainly change the branch lengths and the values of some of the other model parameters, due to more effective estimates of saturation of the nucleotide substitutions (Galtier, 2001). The model is also of much recent interest in functional studies (Gaucher *et al.*, 2002), where it is sometimes called site-specific rate shifts, covarion-like behaviour, heterotachy, or the non-homogeneous gamma model.

A problem with the practical application of these types of models is that the parameters of complex models such as GTR + I + Γ (Posada and Crandall, 2001a) and covarion (Galtier, 2001) require large numbers of nucleotides and large numbers of taxa (e.g. 20–30 sequences) for accurate estimation, as they also do even to differentiate adequately between the models (Sullivan *et al.*, 1999). Increased complexity of models can lead to increased accuracy (because the model fits the data better), but will also lead to increased variance of the parameter estimates, which decreases precision (because there are extra parameters to estimate with the same limited amount of data) — that is, there is a trade-off between systematic error (accuracy) and stochastic error (repeatability). Also, sometimes the extra parameters are simply fitting variation in the data caused by irrelevant factors (e.g. random noise), and so there is no gain in phylogenetic accuracy. Clearly, this will be a problem if only a small number of taxa have been included in an analysis and/or large amounts of data have been excluded as ambiguous.

5.2. Testing Nucleotide Models

Some of the possible tests for violation of the various model assumptions are listed in Figure 5. An important point to keep in mind when evaluating the test results is not whether the data violate the assumptions of the model, but whether any violation is great enough to cause problems for getting the true phylogenetic tree under realistic conditions. The more robust is an analysis, the more it will allow violation of its assumptions. Sadly, we still know far too little about how robust phylogenetic analyses really are.

The stationary models are usually tested as a group (row 1 in Figure 5). That is, the 56 models listed in Table 1 are tested by comparing simpler models to more complex models in an attempt to find the simplest model that adequately fits the data (Posada, 2001; Posada and Crandall, 2001a). The criteria that can be used for comparing models include: invariant tests (Rzhetsky and Nei, 1995), likelihood-ratio tests (Huelsenbeck and Crandall, 1997), the Akaike information criterion (Buckley *et al.*, 2002), and decision theory (Minin *et al.*, 2003). The latter three criteria are expected to be in general agreement about which model is best, but there will be differences in detail. The likelihood-ratio tests require that the models to be tested are nested, which restricts the possible comparisons that can be made, but the Akaike information criterion and decision theory can be applied to all of the tests simultaneously, and this gives them a theoretical advantage (Posada and Buckley, 2004). The likelihood tests are also sensitive to sample size (as are all probability tests), in the sense that a particular model may be rejected when a long sequence is used but not for a shorter sequence length.

There is also a limit to likelihood-ratio testing that arises from the order in which the models are tested (Cunningham *et al.*, 1998). Current computer implementations of these tests (e.g. Posada and Crandall, 1998) arrange them in a pre-specified order, and the final choice of model can be determined by that order because it determines which parameters are tested in conjunction with other parameters. An obvious example that is frequently encountered is choosing to include either a parameter for a proportion of invariant sites or a parameter for a gamma rates-across-sites depending on which of these is

tested first. This can have an effect on the subsequent tree-building (Cunningham *et al.*, 1998; Posada, 2001; Posada and Crandall, 2001a; Pol, 2004). These problems can be by-passed by using model averaging based on the Akaike information criterion (Posada and Buckley, 2004), or by adopting the decision-theoretic approach (Abdo *et al.*, 2004).

The use of Bayes factors to compare models (Suchard *et al.*, 2001; Huelsenbeck *et al.*, 2004; Lartillot and Philippe, 2004; Nylander *et al.*, 2004; Brandley *et al.*, 2005) may alleviate some of the limitations of these tests (because they use the average likelihood rather than the maximum likelihood and so are not affected by tree topology), but this only applies when using a Bayesian tree-building analysis (see Section 6.2). Furthermore, testing the overall adequacy of a model is a separate issue from choosing which of a given range of models is best (i.e. assessment \neq selection). After all, the best model may still be an inadequate description of the data at hand, and if we proceed with the data analysis then we are settling for the best available model rather than the best possible model (Huelsenbeck and Crandall, 1997). As a test of model adequacy, it is usually suggested to use simulations under the proposed model, in which case there are tests for both maximum-likelihood analysis (Goldman, 1993) and Bayesian analysis (Bollback, 2002). These tests are likely to indicate that the simple single-nucleotide models are not yet adequate for real data. Probably, the major factors missing from the models are effects due to genetic selection, since the current models effectively assume that selection is neutral.

Testing the stationarity of the models (row 2 of Figure 5) has been a much more *ad hoc* affair than has been the testing of other aspects of the models. The tests available for examining stationarity of nucleotide frequencies (column 1 of Figure 5) have been reviewed by Jermini *et al.* (2004), and include: testing the compositional stationarity of a specified group of sequences via a contingency χ^2 test; comparing the nucleotide composition of each sequence to that expected under the evolutionary model using a goodness-of-fit χ^2 test; the disparity index (Kumar and Gadagkar, 2001); randomization testing (Siddall, 2001); and the matched pairs test (Tavaré, 1986). The most commonly used of these are the χ^2 tests, but these will be

somewhat biased because they do not take into account correlation due to the evolutionary relatedness of the taxa. In particular, there will be bias unless the tests are based only on the variable sites (Kumar and Gadagkar, 2001). Unfortunately, we do not know which are the variable sites in any given set of sequences, as these will include those sites that are observed to vary and some of those that are observed to be constant. So, the correct result will be somewhere between those from tests both with and without the constant sites included. (Alternatively, and best, one could estimate the proportion of invariant sites, delete the appropriate number of constant sites in proportion to the nucleotide content of the constant sites, and then perform the χ^2 test; but no currently available computer program does this.)

Nucleotide composition (e.g. GC-bias) has been one of the most-studied aspects of non-stationarity (Mooers and Holmes, 2000; Jermini *et al.*, 2004) since it first came to prominence as a problem in phylogeny reconstruction more than 15 years ago (Saccone *et al.*, 1989; Weisburg *et al.*, 1989). Originally, distance-based methods were devised to help deal with the problem (Lake, 1994; Lockhart *et al.*, 1994; Galtier and Gouy, 1995), although likelihood methods were subsequently developed (Yang and Roberts, 1995; Galtier and Gouy, 1998). None of these methods guarantees to successfully deal with GC-bias (Foster and Hickey, 1999; Tarrio *et al.*, 2000; Conant and Lewis, 2001; Rosenberg and Kumar, 2003), possibly because of poor treatment of any concomitant positional variability (e.g. a gamma-distributed rate correction cannot be applied to the distance-based methods). In fact, among-site rate variation is always poorly dealt with by distance methods, even those where it is possible to make some adjustment (Felsenstein, 2001). Any GC-bias may itself cause amino acid bias that also confounds tree-building based on protein sequences (Foster and Hickey, 1999).

The stationarity of nucleotide substitutions (column 2 of Figure 5) can be tested in various ways. Reversibility of the nucleotide-substitution model can be examined using the symmetry goodness-of-fit χ^2 test (Waddell and Steel, 1997), which is basically the matched-pairs test referred to above. The assumption of reversibility reduces the number of model parameters (by half), and it is also the key to

using outgroup rooting (so that we only need to search among unrooted trees not rooted ones). It is therefore a useful assumption to make, but it needs to be checked, as it should probably be treated with scepticism for real data. Constancy of the nucleotide-substitution model can be tested using a triplet Markov analysis (Lin *et al.*, 2002), but this usually requires very long sequences. So, Morrison *et al.* (2004) suggested testing the constancy of the nucleotide substitutions using a heuristic goodness-of-fit χ^2 test, comparing the observed number of substitutions for two monophyletic subsets of the taxa.

Differences between lineages in among-site rate variation (column 3 of Figure 5) can be tested in several ways. The approximate likelihood-ratio test of Huelsenbeck (2002) tests the overall phylogeny by comparing a phylogenetic analysis conducted using the covariotide model with an analysis conducted without it. The inequality test of Lockhart *et al.* (1998) and Steel *et al.* (2000b) directly compares two specified groups of taxa (preferably monophyletic) with each other, without needing a prior phylogenetic analysis. This latter test has been extended by Ané *et al.* (2005).

All of this testing may seem to be rather overwhelming at this point. However, it will hopefully become clearer when the practical issues of testing the adequacy of phylogenetic models are dealt with in Section 8.4.

5.3. Other Aspects of Models

You will note that all of the models discussed above for tree-building are solely about modelling substitutions –in other words, evolutionary models = models of nucleotide substitution. That is, indels (insertions and deletions) are not explicitly modelled at all, and therefore the evolutionary information from indels is effectively ignored. We currently deal with indels by putting gaps into the sequences as a separate procedure (called multiple alignment) and then treat the gaps as missing data when building the tree. So, in spite of the fact that indels and substitutions are both evolutionary events that affect sequences, we model one and ignore the other.

This is probably an incorrect thing to do, not least because of possible detrimental effects of high numbers of missing characters, such as increasing the number of optimal trees found and uncertainty about the placement of some of the taxa (Wilkinson, 1995b; Wiens, 1998; Kearney, 2002). Furthermore, deleting gaps from an alignment before analysis can be seen as simply an attempt to remove indels from the model, leaving only substitutions. That is, the purpose of the multiple alignment procedure seems (in practice) to be to separate the indels and substitutions from each other, so that we can throw the indel information away.

This apparently contradictory behaviour comes, at least partly, from recognizing nucleotides (or amino acids) as the "characters" in sequence data. It is straightforward to treat substitution events when nucleotides are the characters, because these events affect only one character at a time. However, it is hard to treat indels as characters from this perspective, because when an indel event occurs it may simultaneously affect more than one nucleotide position in a sequence. Thus, indel "characters" and substitution "characters" are actually quite different things, and it is hard to create a model that incorporates both of them. So, the problem with indels in a model is that multiple-base indels span >1 substitutional "character," and so the model cannot be a simple extension of our current models. We therefore need to work out how to balance the modelling of gaps versus residues, representing indels and substitutions, respectively. Unfortunately, insertion and deletions may result from two different sorts of events (Li, 1997), with slipped-strand mispairing for short indels and unequal crossing-over or transposition for longer ones (e.g. >30 bases); and so an indel model may not be straightforward to develop.

If indels are incorporated into the model as individual evolutionary events, then we can go straight from the sequences to the tree without the necessity of an intermediate multiple alignment. We can then derive a probabilistic version of the alignment as implied by the tree (i.e. by inferring ancestors) if we want it. Quite a number of people have had a go at addressing this issue, for both likelihood models (e.g. Miklós *et al.*, 2004; Fleissner *et al.*, 2005; Lunter *et al.*, 2005; Redelings and Suchard, 2005) and parsimony (Wheeler, 1996, 1999; Lutzoni *et al.*, 2000). Unfortunately, these methods are either cumbersome or are

currently limited to small data sets, and there are also potential theoretical disadvantages to this approach as well (Simmons, 2004).

You might also have noted that none of the above discussion of models talks about branch lengths. These are part of the tree model rather than the substitution model. These days, it is standard for all likelihood tree-building analyses to allow the branch lengths to vary independently of each other, so that each branch length is a separate parameter in the model (Nei *et al.*, 1998), except in molecular-clock analyses (where the branch lengths must be constrained, by definition). The main thing worth pointing out is the idea that one might not want completely independent branches when combining multiple data sets into a single analysis. For example, if there are two data sets (e.g. different genes), then there are actually three ways of dealing with the tree model when combining them (e.g. trying to get the species tree from the two genes): assume that both data sets have the same branch lengths; assume that both data sets have independent branch lengths; and assume that the two sets of branch lengths are in proportion to each other (i.e. one set is simply a multiple of the other). The latter model is becoming more widely used (Yang, 1996b; Pupko *et al.*, 2002), because it assumes that the underlying tree is common to all of the data sets while allowing different rates of evolution in the different data sets. That is, the evolutionary rate along each branch is assumed to be the product of two rates, one specific to the particular data set (unique) and one specific to the lineage (common), which is a valuable model when searching for the tree that is common to all of the data sets. This is discussed further in Section 7.1. However, it is important to note here that we currently know very little about the effect on tree-building of branch lengths that are unequal or have unequal rates between data sets, and there are clearly circumstances under which likelihood-based methods do not perform well (Kolaczowski and Thornton, 2004; Gadagkar and Kumar, 2005; Mar *et al.*, 2005; Mossel and Vigoda, 2005; Philippe *et al.*, 2005; Spencer *et al.*, 2005).

5.4. Other Types of Models

Protein sequences are sometimes preferred for molecular analyses because the extra number of character states (20 versus 4) reduces the

probability of problems due to homoplasy, particularly parallelisms and convergences, which may be advantageous when analyzing distantly related taxa. There is no necessity for protein modelling to be any different from nucleotide modelling, and yet in practice it has been so. Empirical models of substitution are usually used in protein sequence analysis because the large alphabet of amino acids (20 letters) requires many parameters to be estimated. So, the usual approach has been simply to delve into the databases in order to estimate an "average" substitution rate for the interchanges among each of the 20 amino acids. This average is then used as part of a fixed substitution matrix. It would be interesting to review such models here, but space limitations prevent it.

Since evolution usually occurs at the level of the DNA rather than the amino acid, it is probably more appropriate to develop models of sequence evolution at this level. Thus, more complex models than those discussed in the Section 5.1 have also been proposed for nucleotides, in order to explicitly take into account their role in coding for proteins and RNAs. These models all attempt to be more realistic by incorporating other known biological phenomena into the model. For example, all of the models that I have discussed so far assume that nucleotide positions vary independently of each other, which is an unlikely assumption given the known secondary-structure constraints induced by the function of the coded products. It is worth reviewing some of these ideas because developing more realistic models may be the way of the future.

Spatial non-independence occurs, for example, in adjacent nucleotides that do not mutate independently of each other, which are then referred to as dinucleotides. One way to take this into account is to use the autocorrelated gamma model (Yang, 1995) or the site-specific model (Felsenstein and Churchill, 1996), in which the substitution rate at one site depends to some extent on the rate at an adjacent site. Alternatively, full doublet models have been developed (Schöniger and von Haeseler, 1994; von Haeseler and Schöniger, 1998; Jensen and Pedersen, 2000; Arndt *et al.*, 2003; Lunter and Hein, 2004; Siepel and Haussler, 2004), in which there are 16 possible character states (one per dinucleotide) instead of four (one per nucleotide), and thus 120 possible changes between states (i.e. substitutions) instead of six.

No consensus seems to have been reached about the best approach to take to developing these models, however, and so none has come into common use in spite of their obvious potential.

For protein-coding sequences, various codon-based models have been developed (Muse and Gaut, 1994; Goldman and Yang, 1994; Halpern and Bruno, 1998), which allow for evolutionary dependency among sites within codons. For example, if there is selection for a particular amino acid at a site in a polypeptide, such as a functional constraint, then the nucleotides in the relevant codon cannot evolve independently of each other. The models work by having 61 possible character states (one per codon) instead of four (one per nucleotide), which clearly increases the computational effort. Furthermore, this would result in 1830 possible changes between character states instead of six, which means that simplifying assumptions need to be made, such as making the distinction only between non-synonymous and synonymous nucleotide substitutions or between amino acids with different biochemical characteristics (Ren *et al.*, 2005). Some of these ideas have been further explored, including the incorporation of GC-bias (Pedersen *et al.*, 1998), among-site variability (Nielsen and Yang, 1998; Yang *et al.*, 2000; Kosakovsky Pond and Muse, 2005) and some aspects of non-stationarity (Yang and Nielsen, 2002).

Various models have been developed that take into account non-independence of nucleotide positions that are paired in helices of RNA-coding sequences (Muse, 1995; Rzhetsky, 1995; Tillier, 1994; Tillier and Collins, 1995, 1998; Knudsen and Hein, 1999; Schöniger and von Haeseler, 1999). These stems and their associated single-stranded loops are the sites of catalytic activity in the RNA, and so compensatory substitutions are required in the complementary strands in order to keep the stable helix structure. These models have either 6, 7 or 16 possible character states, depending on how many states are used for the mismatched (i.e. non-pairing) nucleotide doublets, but the more general models perform better than the simpler ones (Savill *et al.*, 2001). Accounting for essential parameters such as rate heterogeneity is still somewhat of a problem, though.

There is also, intriguingly, the possibility of developing models for sequences that simultaneously code for both proteins and functional

RNAs (Pedersen *et al.*, 2004), such as occurs for example in some viruses.

While the models discussed in this section are available in some computer packages, and have been successfully applied in a Bayesian context (Jow *et al.*, 2002; Telford *et al.*, 2005), none of them has yet become popular. This may be due to some technical limitations or an unrealistic assumption, although the extra computational effort should not be overlooked (Ren *et al.*, 2005). Furthermore, it is not yet straightforward to test the adequacy of these models (e.g. Kjer, 2004).

6. TREE-BUILDING

Perhaps the most commonly used tree-building method in molecular genetics is neighbour-joining, while in systematics it probably is maximum parsimony. Preference for the latter method is often argued from a philosophical point of view (e.g. Siddall and Kluge, 1997; Grant and Kluge, 2003). However, from the point of view of an evolutionary model, as discussed in the previous sections, it can actually be one of the most complicated methods (Tuffley and Steel, 1998a), although simple model formulations do also exist (Goldman, 1990; Penny *et al.*, 1994). The neighbour-joining tree-building method seems to be employed solely on the grounds of computational speed (Hollich *et al.*, 2005). This criterion may have some value in a heuristic evaluation of the data, but it should not be taken too seriously in any attempt to reconstruct the true phylogenetic tree (Williams and Moret, 2003; Hall, 2005).

Since enough has been written about these two methods, there is no need for me to discuss them further here. Instead, I will mention some points with regard to tree-building using the sorts of likelihood models that I have discussed in Section 5, with emphasis on explaining the practical problems involved. As far as likelihood models are concerned, there are two basic approaches for optimizing the tree in relation to the model: maximum likelihood and Bayesian posterior probability. For a well-defined model and data with a strong phylogenetic signal these two approaches should indicate the same tree as

being the optimal one, but not necessarily otherwise (Williams and Moret, 2003; Hall, 2005; Mar *et al.*, 2005).

The basic idea is that we have a likelihood function, which is an equation that describes how to calculate the likelihood of a particular phylogenetic tree topology given values for the combination of branch lengths and substitution-model parameters. This function allows us to evaluate any given tree quantitatively, to give it a score (a likelihood value, which is analogous to a probability) that describes the fit of the tree in relation to the model and the data. We then need a criterion for how to use these likelihood values to decide which tree is the optimal one. For the maximum-likelihood method, we choose the tree that has the largest observed likelihood value (that is, the one that maximizes the probability of the data given the model), while for Bayesian analysis we choose the tree that has the largest total likelihood (that is, the largest posterior probability). Mathematically, the maximum-likelihood method finds the maximum (over all possible trees) of the relative likelihood (measured for each tree), while the Bayesian method finds the maximum (over all possible trees) of the integrated likelihood (measured for each tree) (see Steel and Penny, 2000). Usually, the tree found by the former is called the maximum-likelihood tree while the tree found by the latter is called the maximum a posteriori (MAP) tree.

6.1. Maximum Likelihood

Currently, the most popular method for incorporating likelihood models into phylogenetic analysis is via the maximum-likelihood method (see Huelsenbeck and Crandall, 1997; Lewis, 1998a; Sanderson and Kim, 2000). This approach to likelihood has a sound and long-standing basis in statistical methodology, which is its main strength. However, it is a very computer-intensive approach to tree-building, which is its main practical weakness.

The practical problem with the current implementations of maximum-likelihood tree-building is the double optimization that is needed, which results from the particular way that likelihood has been applied in phylogenetic analysis (Yang *et al.*, 1995). An analytical

solution to maximum-likelihood estimation only exists for the very simplest case (i.e. a rooted tree for three taxa based on binary characters with symmetric substitutions and a molecular clock; Yang, 2000), and so an iterative procedure is required for most data sets. First, the branch lengths, and also the other model parameters unless these have been fixed, are optimized in order to find the maximum-likelihood value for a particular tree topology. Second, tree topologies are searched to find the tree with the largest maximum-likelihood value. Both optimizations are needed in maximum-parsimony tree-building as well, but the first optimization step is considerably more complicated for maximum-likelihood tree-building (described by Sullivan *et al.*, 1999).

The basic issue with the first optimization is that increasing complexity of the evolutionary models (as discussed in Section 5) results in an increasing number of calculations, often exponentially so. This makes a process that is slow at the best of times even more impractical. Various strategies have been developed to try to speed up the calculations for the first optimization (Kosakovsky Pond and Muse, 2004), some of which result in approximate likelihood estimates that can be used to eliminate unpromising trees, including branch-length approximations (Rogers and Swofford, 1998), parameter approximations (Gu and Zhang, 1997), subtree equality vectors (Stamatakis *et al.*, 2002) and distance-based likelihood approximations (Woodhams and Hendy, 2004). Perhaps the most commonly used method in practice is to fix the substitution-model parameters for the duration of the analysis, having estimated these at the same time as choosing the model, thus leaving only the branch lengths to be optimized for each tree. However, this is not very successful at finding the desired tree. A better method is to use a successive approximations approach, which iterates between estimating the parameter values of the substitution model (with the tree fixed) and searching for trees (with the substitution model fixed) (Sullivan *et al.*, 2005). This usually takes only 3–4 iterations to converge to a solution, but the strategy seems to be just as successful as a full search in which the substitution-model parameters are optimized for each tree, and it takes only a small fraction of the time.

The maximum-likelihood approach has been shown to be NP-complete under a variety of conditions (Addario-Berry *et al.*, 2004;

Chor and Tuller, 2005a, b), which means that it will be impossible to guarantee to find the tree topology with the largest maximum-likelihood value (i.e. the second optimization) other than by checking all possible trees. Branch-and-bound methods (which guarantee to find the global optimum but do not necessarily have to search through all of the trees) are not yet known, although some attempts have been made to provide bounds (Hendy and Holland, 2003) or to develop maximum-likelihood methods that might be amenable to this approach (Woodhams and Hendy, 2004). Alternatively, parallel computing has been successfully applied to the tree searches (Ceron *et al.*, 1998; Katoh *et al.*, 2001; Stewart *et al.*, 2001; Schmidt *et al.*, 2002; Keane *et al.*, 2005; Minh *et al.*, 2005; Reeves *et al.*, 2005) and to the parameter estimation (Schmidt *et al.*, 2003), which will help speed things up.

Nevertheless, the most commonly used strategy at the moment involves heuristic searches, which cannot guarantee to find the global optimum. The most popular computer programs use simple "hill-climbing" algorithms (Swofford *et al.*, 1996). This approach consists of finding a "good" starting tree and then swapping branches to see if a better tree exists. This can be an effective strategy, but it is inefficient because most of the analysis time is spent evaluating the likelihood of trees that are very suboptimal, and this inefficiency becomes exponentially worse as the number of taxa increases. Furthermore, the time required to complete a heuristic search cannot be estimated for any particular data set, which is a severe limitation when planning an analysis. The time that an analysis takes will depend on: how much incongruence there is among the characters in the data set (which determines how many nearly optimal trees there are to search through), the shape of the tree that is used to start the branch swapping (which determines the number of rearrangements that can be made), and what proportion of the potential rearrangements will be performed before a better tree is found (which determines how much time is "wasted"). There is no simple mathematical formula for calculating these dependencies.

Guindon and Gascuel (2003) have proposed a hill-climbing method that optimizes both the topology and the branch lengths simultaneously, thus trying to avoid local minima early in the tree search. This

seems to speed up calculations considerably. Stamatakis *et al.* (2005) have provided an algorithm that performs limited forms of branch swapping on an initial parsimony tree, and this seems to be another competitive approach in terms of time. Alternative computational strategies have also recently been explored. These include quartet puzzling (Strimmer and von Haeseler, 1996; Vinh and von Haeseler, 2004), genetic algorithms (Lewis, 1998b; Katoh *et al.*, 2001; Brauer *et al.*, 2002; Lemmon and Milinkovitch, 2002; Jobb *et al.*, 2004), simulated annealing (Salter and Pearl, 2001), reweighted tree perturbations (Vos, 2003), divide-and-conquer frameworks (Du *et al.*, 2005) and even hybrid distance-likelihood methods (Ota and Li, 2000, 2001; Ranwez and Gascuel, 2002). None of these approaches has become popular as yet, perhaps because all they really do is provide a possibly suboptimal answer faster. Moreover, the programs implementing these methods often provide only a subset of the nucleotide-substitution models, which limits their generality. Indeed, many of the claims of superiority refer only to the large number of taxa that can be accommodated in the analysis rather than to the optimality of the solution found.

Another potential problem with the use of maximum likelihood as a criterion, which has not been sufficiently emphasized in the general literature, is that the maximum-likelihood tree (if it is found) may be only slightly more optimal than a large set of other trees. This means that an incredibly small difference in the estimated-likelihood value for a tree may determine its choice (Williams and Moret, 2003). This may not be a problem if the model has been specified correctly, but if it has not been then the analysis will fail, because the true tree will be among the slightly suboptimal trees but only the tree estimated to be optimal will be reported. It has therefore been emphasized in the literature that models need to be sufficiently complex—that is, they need to have enough parameters to adequately describe the data. However, the analysis will also be sensitive to over-parameterization (or over-fitting) of the model—that is, having too many parameters in the model relative to the amount of data, so that you cannot identify the optimal value for some of them (Rannala, 2002). This leads to variance inflation due to correlation among the parameter estimates, resulting in unreliable estimates of the parameter values. This is a

general problem when using likelihood, but it can be a particular problem for maximization algorithms because of mis-estimation of the maxima.

Thus, you need to be wary of model specification in a maximum-likelihood analysis. The result is determined by the estimation of which tree (or trees) has the maximum likelihood, and any slight mis-estimation due to mis-specification of the model can affect which tree is reported as the optimal one. These differences need not be statistically significant, but that is not the point—the maximum-likelihood criterion specifies only the maximum likelihood as the objective and does not give credence to suboptimal likelihoods, unless you do this for yourself.

6.2. Bayesian Analysis

Bayesian analysis has only recently been proposed as a method for incorporating likelihood models into phylogenetic analysis (reviewed by Huelsenbeck *et al.*, 2001, 2002b; Holder and Lewis, 2003). However, this approach to data analysis also has a sound and long-standing basis in statistical methodology, which offers it the same strengths as maximum likelihood. Moreover, although it is a computer-intensive approach to data analysis it may be more practical, and so it is becoming widely used throughout biology as a whole (Basáñez *et al.*, 2004; Beaumont and Rannala, 2004).

Bayesian analysis uses the same evolutionary models as does maximum-likelihood analysis and it also uses the likelihood function to quantify the trees, but it assesses the choice of optimal tree differently. The optimal tree in Bayesian analysis is the one that has the maximum posterior probability. This probability is measured for each tree as a combination of the prior probability of the tree, the likelihood of the data given the model and the tree, and a standardization or normalizing constant (to make the probabilities sum to 1). One way to think about the difference between maximum-likelihood and Bayesian analysis is that, when assessing a particular tree, in the first case we are trying to find the combination of parameter values that produces the greatest likelihood, while in the second case we are trying to find

the average likelihood across all of the possible parameter values. Mathematically, this means that maximum likelihood uses the profile likelihood while Bayesian analysis uses the integrated likelihood.

The practical problem with implementation of Bayesian tree-building is that the standardization constant, which measures the probability of the data, involves a summation over all possible trees, and then for each tree an integration over all of the model parameters and branch lengths. This leaves us in roughly the same sort of situation as exists for maximum likelihood (i.e. it is nigh on impossible for most data sets), and so a heuristic technique is used here as well. This technique is the Metropolis–Hastings version of Markov chain Monte Carlo (MCMC), which basically takes a directed sample of the trees that are likely to have the largest posterior probability (see Lewis, 2001b for a simple conceptual analogy). If the sampling is performed correctly (see below) and the sample is large enough (we are taking a large dependent sample rather than a small independent sample, as would be more usual in science), then this approach will produce a good approximation to the desired answer (i.e. we will have an estimate of the posterior probabilities for all of the trees with the highest probabilities).

To see if the sampling has been performed correctly, we need to check both the convergence and the mixing of the Markov chain (i.e. the sequence of samples). Convergence refers to whether the samples have been consistently taken from areas with high likelihood (i.e. we have passed the “burn-in” period where the Markov chain has not yet got to the desired probability distribution), while mixing refers to how well the Markov chain has sampled the possible parameter values (i.e. we have included all of the different types of trees with similar likelihoods). The usual suggestion for checking convergence and mixing is to plot the estimated likelihood and parameter values during the course of the Markov chain, to observe the behaviour of the chain. Furthermore, it is best to run several analyses, each from a different random starting tree, and see whether the resulting likelihoods and parameter estimates are different—if they are, then the Markov chain was not run for long enough.

An example of the sort of problem that may be encountered is shown in Figure 6. The data for the analysis are discussed in Section 8,

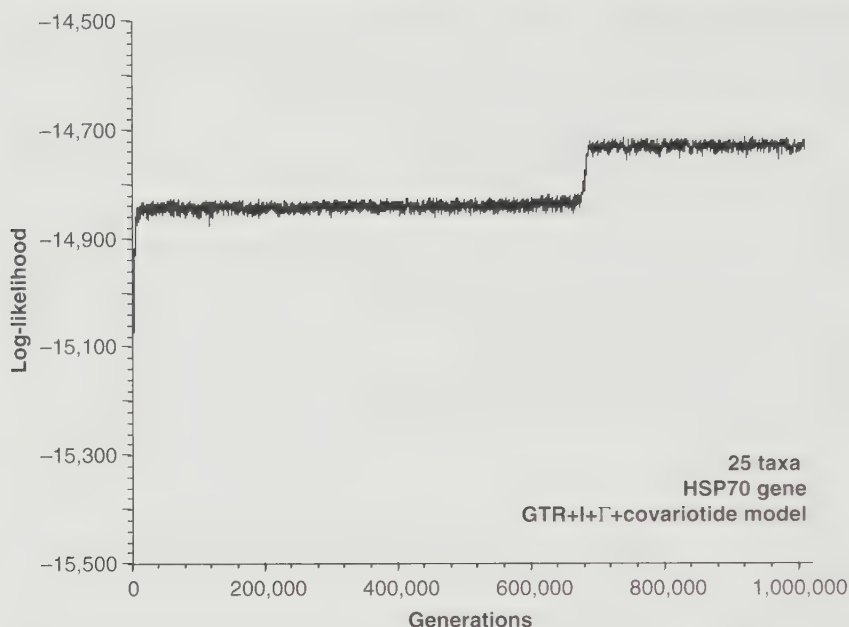


Figure 6 Plot of the calculated log-likelihood for the phylogenetic tree at each sampled generation (sampling every 100 generations) of the 1 010 000 generations of the MCMC Bayesian analysis of the data for the *Cryptosporidium* HSP70 gene. The data and the analyses are discussed in Section 8. The data for the graph were analyzed using the GTR + I + Γ + covariotide substitution model. The likelihoods for the first 20 sampled generations cannot be shown on this scale, as they start at a log-likelihood of -22 901.

consisting of sequence data for the HSP70 gene of some samples of *Cryptosporidium* plus several other Apicomplexa as an outgroup. An example of Bayesian analysis (using the MrBayes version 3.0b4 program; Ronquist and Huelsenbeck, 2003) with a complex substitution model is shown. Each step of a Markov chain is called a generation (i.e. a combination of tree topology and parameter values), and it is usual to take a sample from the chain at set intervals, typically every 100 generations. The first series of samples will have small likelihoods (because the chain starts from a randomly chosen tree), and the samples taken during this period are discarded as burn-in. Only part of this stage of the MCMC chain is shown in the graph, as the first few likelihood values are very small. However, after about 10 000

generations the chain seems to converge on a likelihood value of $c. -14840$, and it continues in this way for nearly another 670 000 generations. (NB. This value is actually a log-likelihood, which is the natural logarithm of a probability, so that the values are negative and approach 0 as the probability approaches 1.) However, at that stage the likelihood rapidly increases to $c. -14730$, where it remains until the chain was stopped (after 10 000 samples had been taken). The graph thus makes it clear that the stationary distribution was not reached until the chain had been running for a very long time – some people only run 500 000 generations, and in this case they would not have been sampling from the desired posterior distribution of trees at all. The cause of the change in this particular example is the tree topology with respect to the outgroup. The tree topologies sampled first in the chain had the outgroup as a single group, while the change in likelihoods was associated with the “discovery” of a set of topologies that had two of the outgroup taxa, *Toxoplasma gondii* + *Babesia bovis*, in one place on the tree and *Plasmodium falciparum* in another (see Section 8.4).

Thus, it is important to note that plotting the likelihood values as the chain progresses is insufficient to assess convergence, and it will provide only a minimum estimate of the burn-in time – the other parameter estimates need to be plotted as well. Furthermore, it will usually be best to plot the data for several chains, particularly for large data sets (in terms of both taxa and characters) and for complex evolutionary models. Adjustment of the “tuning parameters” of the computer program may be helpful, as this may improve the mixing of the chain, which will lead to more rapid convergence. Finally, for safety it may be best to increase the burn-in period somewhat (e.g. 10%) beyond apparent convergence.

The second major issue with Bayesian analysis is the need to explicitly specify prior probabilities (usually abbreviated as priors) for all of the model parameters. This includes specifying priors for the tree topology and branch lengths as well as for all of the parts of the substitution model (e.g. nucleotide frequencies, substitution rates, proportion of invariant sites, etc). In an ideal phylogenetic analysis, the values chosen for the priors will have little effect on the resulting tree inferences, because the posterior probabilities will be dominated

by the strength of the phylogenetic patterns in the data. However, it is becoming increasingly clear that the values assigned to these priors can have a significant effect on the resulting tree topology, branch lengths and clade support (Zwickl and Holder, 2004; Yang and Rannala, 2005).

This means that we have to be careful as to exactly how we choose to specify the priors. In the absence of any pre-existing information for our taxa that could help us make the decisions, it is traditional to use what are called uninformative priors. These attempt to minimize the effect of the priors on the posterior probabilities, allowing the data to dominate the tree inferences. Unfortunately, this is more difficult than it sounds, as there are competing influences within the complex tree-building procedure that mean that it is almost impossible to specify a set of priors that are uninformative with respect to all aspects of the procedure. At the moment, the available computer programs have default values for the priors that are rarely questioned by the users. Since there has been little quantitative research yet carried out on the effect of mis-specified priors, it is unclear whether these default values are appropriate or not. Presumably, this will be an area of active research over the next few years.

In the meantime, a simple example will make clear the general problem that occurs when trying to decide how to specify prior probabilities in a phylogenetic analysis. One of the priors that we need to specify is the probability associated with each possible tree shape that could exist for our set of taxa. For a set of six taxa there are 105 possible unrooted trees. If we want an uninformative prior, then we might choose to make each unrooted tree equally likely *a priori*, and this is called a uniform or flat prior. However, if we remove the labels from the unrooted trees, then it is obvious that there are only two different topologies, as shown in Figure 7. There are 15 unrooted trees associated with the topology on the left and 90 unrooted trees associated with the topology on the right. So, what do we want our uninformative prior to be associated with: the unrooted labelled trees or the unlabelled topologies? If we make the unrooted trees equally likely (i.e. uninformative), then we are making one of the topologies six times as likely as the other (i.e. informative). If we make the two topologies equally likely, then we make some unrooted

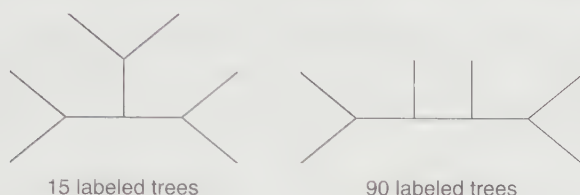


Figure 7 The two unrooted unlabelled tree topologies for six taxa. If the terminal branches are labelled, then there are 15 possible unrooted trees for the topology on the left, and 90 possible unrooted trees for the topology on the right.

trees much more likely than others. We simply cannot have a prior that is uninformative with respect to both characteristics simultaneously (i.e. there is no unique choice of a uniform prior). Furthermore, a similar statement can be made with respect to topologies and clades on rooted trees (Pickett and Randle, 2005). Indeed, it was the use of a uniform prior that was the principal objection of Fisher (1922) to the method of Bayes (1763), when advocating his own method of maximum likelihood, as he considered that the non-uniqueness of uniform priors made them arbitrary. Current computer programs usually make the unrooted trees equally likely rather than their topologies, but we have no idea whether this is the most appropriate choice or not (c.f. Stone, 1998). It does, however, make the MAP tree the same as the maximum integrated likelihood tree, so that we maximize our average chance of finding the correct tree (Steel and Penny, 2000).

Thus, the key to successfully running a Bayesian analysis is not just choosing a suitable evolutionary model, but also involves choosing priors and making sure that the Markov chain is doing its job. There is currently no such thing as a standardized Bayesian analysis that we can all agree upon as the best option. This adds a responsibility for the user that does not necessarily exist for maximum-likelihood analyses, although devising a suitable heuristic search strategy for that type of analysis is not necessarily straightforward either (see Sanderson and Shaffer, 2002). However, the particular heuristic strategy used for Bayesian analysis gives it three practical advantages over maximum-likelihood analysis: (i) the time that an analysis will take can be estimated accurately; (ii) more complex evolutionary models do not

dramatically increase the time taken for analysis and may not increase it at all; and (iii) estimation of the degree to which each branch is supported by the data (and model) is provided as an integral part of the analysis.

Advantage (i) occurs because we specify how long the analysis should take by choosing the number of generations that the Markov chain should run. It may take several trial runs with a particular data set and the proposed model to estimate this time, but it cannot be estimated at all for heuristic maximum-likelihood analyses. Moreover, parallel computing has also been successfully applied to Bayesian tree-building analysis (Altekar *et al.*, 2004), which will speed things up considerably. Advantage (iii) comes from the fact that at the end of the analysis we have an estimate of the posterior probability of each tree and which branches it contains. The proportion of the trees containing each branch of interest is referred to as the clade credibility (Rannala and Yang, 1996; Larget and Simon, 1999), and this is a natural measure of branch support. Thus, we do not need to run a lengthy bootstrap analysis after we have found our optimal tree. The relationship between bootstrap values and clade credibilities is a complex one (see Section 3.5 and Yang and Rannala, 2005).

Advantage (ii) stems from the fact that only the marginal distributions of the parameters are required for MCMC calculations rather than the joint distributions, and so the parameter values are simply monitored as the Markov chain progresses (i.e. each parameter can be recorded independently of the others). It takes little more time to monitor the values of a large number of variables than of a small number (although using the gamma distribution to deal with among-site rate heterogeneity does slow down the calculation of the likelihood; Nylander *et al.*, 2004). Moreover, creating complex models by partitioning the data (see Section 7.1) may actually increase the speed of the analysis, because the likelihood of each partition can be calculated more quickly (Nylander *et al.*, 2004). However, increasing model complexity can also be abused. For example, it is possible to over-parameterize models in a Bayesian analysis (Rannala, 2002), because this is a by-product of using the likelihood function. It is likely that this will have less of an effect on a Bayesian analysis than on a maximum-likelihood analysis, and that under-parameterization

will be a much bigger problem in Bayesian analysis than over-parameterization, because the first leads to biased parameter estimates while the second leads only to slight imprecision (Lemmon and Moriarty, 2004; Nylander *et al.*, 2004). Convergence and mixing may be more problematic for more complex models, and these aspects should be checked thoroughly (Nylander *et al.*, 2004).

Note carefully that these arguments in favour of Bayesian analysis are merely practical ones. I am not arguing that Bayesian analysis does or does not also have theoretical advantages, as this is an ongoing debate in the statistics literature. Bayesian analysis is obviously not the answer to our prayers; and its growing use in biology is merely a product of the fact that it offers a heuristic strategy that is fast (or at least predictable) while simultaneously allowing complex biological models to be used. In this sense, we can expect that its use will become even more widespread, irrespective of whether Bayesian probabilities are “better” or “worse” than frequentist probabilities and whether it is better to use profile likelihood or integrated likelihood. Alternatively, we may go in the opposite direction, to what may be called the non-parametric phylogenetic analysis (Sanderson and Kim, 2000), although there is little sign of this so far.

7. COMBINING DATA SETS

It is becoming more common for phylogeneticists to want to combine different sources of evidence when constructing a tree for their taxa (de Queiroz *et al.*, 1995; Brower *et al.*, 1996). For example, they may have data of different types (e.g. molecules and morphology) or different data sets of the same type (e.g. sequences of several different genes). This is simply a recognition of the fact that for a tree to have any chance of representing the evolutionary relationships among species it needs to be based on as many different types of data as possible.

In particular, it has long been recognized that a tree based on a single gene sequence represents only the evolutionary history of that particular gene (Goodman *et al.*, 1979; Pamilo and Nei, 1988). Each gene can have its own individual history, independently of the history of the other genes in the same species, due to various phenomena such

as allelic (or ancestral) polymorphism, introgression, lineage sorting, unequal rates of speciation and gene mutation, lateral (or horizontal) transfer, hybridization or mistaken orthology (Doyle, 1992; Brower *et al.*, 1996). Therefore, constructing a robust and reliable species tree requires combining several gene trees—most genes on their own are poor sampling estimates of the overall genome history (Cummings *et al.*, 1995; Rosenberg, 2002). Furthermore, a living organism is an integrated functioning whole, not just a collection of unrelated genetic attributes. Thus, an organism is a collection of interactions between genes, and between genes and their environment (i.e. a phenotypic whole), and it is the organism as a whole that takes part in the evolutionary process. Consequently, there is no more reason for direct genetic attributes to reflect phylogeny than for anything else to do so (de Queiroz *et al.*, 1995). In fact, morphological characters may be a better reflection because they integrate many genetic and phenotypic characters (Baker *et al.*, 1998).

Note that there are actually two separate issues here. First, a tree produced from any one data set may or may not represent the true history of the taxa in that data set (e.g. your reconstructed gene tree might not be the true gene tree). Second, even if you have the true tree for your data set it still may or may not represent the true history of the taxa (e.g. the gene tree might not be the true species tree). Dealing with both of these issues simultaneously is no mean task, but we need to succeed if we are to obtain the best phylogenetic tree possible. The only guideline we have is congruence among our various empirical results, since we have no direct access to the “truth”—congruence among results is no guarantee of phylogenetic accuracy (because of systematic error; Grant and Kluge, 2003) but it is not a bad thing to have (because it quantifies stochastic error; Cunningham, 1997b). The important point, then, is to explore each of the data sets separately and in combination. That way we can try to understand which data patterns occur in which data sets, and possibly why, as well as which data patterns “emerge” when the data are combined. The latter issue is important because there can be “hidden support” or “hidden conflict” in the data, which will only become clear when combined and separate analyses are compared (Gatesy and Baker, 2005).

There are two basic strategies for analyzing combined data from multiple data sets: (i) combine the data into one set and then produce a single tree from it; and (ii) produce a tree from each of the data sets and then combine these into a single tree. That is, we can do the combining either before or after we do the tree-building. The first strategy I will call concatenation (since we concatenate the data), while the second will be called consensus (since we need a consensus of the trees). However, these strategies have been called many different things in the literature, including combined analysis, simultaneous analysis or supermatrix for (i) and separate analysis for (ii). These two strategies may produce mutually contradictory answers (Barrett *et al.*, 1991), and there is a long history of debate concerning their relative merits (de Queiroz *et al.*, 1995; Brower *et al.*, 1996; Huelsenbeck *et al.*, 1996a), which I will not go into here.

7.1. Concatenation

The concatenation approach combines all of the data for all of the taxa into a single data matrix for the tree-building analysis. From the point of view of modelling, as used in this review, the concatenation approach is usually successful at reducing stochastic error (see Section 2.1), but it can provide strong support for the wrong groups if the evolutionary model used in the analysis is a poor fit to reality (i.e. systematic error). Therefore, it will usually be best to model the different data sets as separate partitions, so that each partition is analyzed with the most appropriate model—it would be naïve to simply concatenate the various data sets and try to analyze the result as a single data set (sometimes called the total evidence approach), because it is unlikely that a single “average” model will just happen to fit the data (although see Buckley *et al.*, 2002; Gontcharov *et al.*, 2004). Even if the different data sets are derived from the same evolutionary history, if they have different evolutionary dynamics (i.e. are based on different processes) then they will yield biased estimates of that history if they are combined and analyzed by a single model that is a poor fit to at least one of the data sets.

Consequently, in a concatenation analysis data sets should only be combined into a single partition if they are not significantly heterogeneous (Bull *et al.*, 1993)—heterogeneity indicates that the evolutionary processes are different enough in the various data sets to produce different estimates of the phylogenetic tree. For likelihood analyses it is best to assess the relative utility of various combinations of partitions, to find out which parts of the data sets can be combined, and then use a composite model if the data cannot all be combined into a single partition. That is, we would combine those parts of the data sets that appear to fit the same evolutionary model, while keeping separate partitions for each of the unique models that we identify, and then perform a simultaneous analysis of the data based on the composite set of models (Yang, 1996b; Pupko *et al.*, 2002; Yang and Swanson, 2002; Nylander *et al.*, 2004; Brandley *et al.*, 2005).

For example, for protein-coding nucleotide sequence data we might consider three partitions, one for each of the three codon positions. If it turns out that the 1st and 2nd positions, say, are adequately fitted by the same model then we would combine them into a single partition, while keeping the 3rd codon positions in a separate partition. A single data analysis would then be performed using the two partitions, each with its own model. Alternatively, if we have data for multiple genes we might consider different partitions for each of the genes. If it turns out that some of the genes are adequately fitted by the same model then we would combine them into a single partition, while keeping the remaining genes in separate partitions. We could also try mixtures of these two ideas, with separate partitions for each codon-position gene combination, or we could use different coding regions within genes, different structural or physical-chemical regions or even different genomic regions (e.g. nuclear versus mitochondrial or chloroplast). Since there are many different possible partitions, the choice of appropriate partitions is difficult, and the logical basis for choosing any particular partitioning scheme must thus be made clear (Chippindale and Wiens, 1994). The basic idea in all cases is to find the minimum number of process partitions required, each with the simplest model that it needs, for an adequate fit to all of the data (DeBry, 1999, 2003; Castoe *et al.*, 2004).

We could test whether the different partitions are significantly heterogeneous using a likelihood-ratio test (Huelsenbeck and Bull, 1996; Waddell *et al.*, 2000), and then combine the homogeneous partitions; or we could use the likelihoods or a likelihood-ratio test to compare different a priori composite models (Yang, 1996b; Castoe *et al.*, 2004), and choose the one with the optimal likelihood; or we could see if the tree from the combined analysis falls within the bootstrap or Bayesian credible intervals of the trees from the separate models (Buckley *et al.*, 2002); or we could formally quantify the fit of different partitioning arrangements using Bayes factors (Brandley *et al.*, 2005); or we could simply find the best-fit model for each a priori partition using the set of tests for stationary models described in Section 5.2, and just use the resulting individual models and partitions. Whichever way we choose to proceed, the best method to produce a single tree from multiple data partitions in a likelihood analysis is probably (Yang, 1996b; Pupko *et al.*, 2002; Yang and Swanson, 2002; Nylander *et al.*, 2004; Brandley *et al.*, 2005) to assume that the data in all of the partitions are evolving on the same tree topology (so that we are trying to estimate the same tree for all of the data) but that the branch lengths are proportional to each other across the partitions (so that we allow different data sets to evolve at different rates) and that the substitution-model parameters are evolving independently in different partitions (so that we can fit the model as closely as possible to each data set). This approach, of using common values for some model parameters (relating to the tree) and partition-dependent values for other parameters (relating to substitutions), is used in the example in Section 8.5.

The main advantage of the concatenation method is that it directly evaluates the original data when producing the final estimate of the combined phylogeny – there is greater information content in simultaneous analysis of the primary data (Lecointre and Deleporte, 2005). For example, suboptimal patterns that are common to all of the data sets, and which might not appear in a tree based on any of the individual data sets, can be detected (Bull *et al.*, 1993).

However, incompatible data types provide one limitation to the approach (e.g. distance-based data cannot be combined with character-state data), congruence among different data sets cannot be

assessed and the imposition of a single optimization criterion may also be limiting (e.g. it is conceivable that we might want to use one optimization criterion for one data set and a different criterion for another data set). Furthermore, partitioned analysis implies increased computational complexity compared to analyzing a single partition, if the data are to be analyzed simultaneously. This is bad for maximum-likelihood analysis (Sanderson and Kim, 2000; DeBry, 2003), and none of the currently available programs allow sophisticated searches using partitioned models. (In fact, currently only the PAML program does it at all; Yang, 1997b.) However, this is where Bayesian analysis comes into its own, as subdividing the data into partitions can actually increase the speed of a Bayesian analysis, because the increased speed associated with analyzing smaller data partitions more than outweighs the decrease in speed associated with increasing the number of partitions (Nylander *et al.*, 2004). Alternatively, for larger data sets various heuristic approaches have been taken to partitioned analysis when using maximum-likelihood tree-building (Caterino *et al.*, 2001; Baptiste *et al.*, 2002; Gontcharov *et al.*, 2004), based on estimating the likelihood of a set of previously obtained trees, although these approaches do not allow proportional branch lengths. Seo *et al.* (2005) provide a method for formally combining likelihoods from trees for different partitions in order to choose the optimal tree.

7.2. Consensus

The consensus approach, on the other hand, analyses each of the data sets separately and then compares the resulting set of trees. From the point of view of modelling, the consensus approach can have poor resolution of the relationships (due to conflict among the trees) and can be subject to stochastic error, but presumably the appropriate model will be used to produce each tree to ensure a reasonable fit to reality. It is not uncommon for researchers to simply compare the set of trees subjectively (i.e. to display all of the trees and then to compare them visually), based on the idea that congruence among the trees is what provides the best evidence for the true species tree.

However, I recommend some form of quantitative procedure for combining the trees into a single tree.

One objective way to combine the multiple trees is via a consensus tree (de Queiroz, 1993). There are quite a number of different strategies that have been developed (reviewed by Swofford, 1991; Bryant, 2003), with strict consensus and majority-rule consensus being far and away the most commonly used (the latter frequently including all other compatible groups as well as the groups occurring in $> 50\%$ of the trees). All of the methods will produce a similar consensus if the source trees are all compatible with each other, but they can differ dramatically if some of the trees are incompatible, and there is no real consensus among researchers as to which consensus method to use under these circumstances. Most of the methods produce a tree that represents the uncontested groupings among the source trees, but an Adams consensus tree can potentially indicate groups that do not occur in any source tree. Even a small amount of incompatibility can result in loss of resolution of the consensus tree, which means that most of the methods are too conservative or are biased (Sumrall *et al.*, 2001). The best method for dealing with lack of resolution is to use reduced (or pruned) trees (Wilkinson, 1994, 1995a), but these sorts of subtree have not yet found wide acceptance (c.f. Figure 4).

If the sampling of taxa is not identical in each of the data sets, but the sampling overlaps between trees (i.e. at least two of the taxa are common between trees), then the resulting tree is called a supertree (Gordon, 1986; Bininda-Emonds *et al.*, 2002). Once again, quite a number of different strategies have been developed (Bininda-Emonds *et al.*, 2002; Bininda-Emonds, 2004). Some of the methods (called agreement supertrees) simply represent the uncontested groupings among the source trees, while others (called optimization supertrees) try to optimize some function describing the fit of the supertree to the source trees. The agreement methods are usually analogues of conventional consensus methods, treating all of the source trees as equal, while the optimization methods are novel methods, usually not combining the source trees equally. Consequently, much of the conservativeness of the conventional consensus methods is lost in the optimization supertree methods, which may explain their increasing popularity. Most of the methods concern themselves solely with the

topology of the tree rather than with the branch lengths, the exception being the average consensus procedure of Lapointe and Cucumel (1997), although some methods have also been developed that allow ancestral divergence dates to be included (Semple *et al.*, 2004). Furthermore, it is important to note that both conventional consensus and supertree methods have mathematical limitations on what is possible if we wish to obtain a single tree (McMorris, 1985; Steel *et al.*, 2000a), so that no entirely satisfactory mathematical method can ever be developed for unrooted trees.

Matrix representation with parsimony (Baum, 1992; Doyle, 1992; Ragan, 1992), usually abbreviated as MRP, is the only commonly used supertree method, apparently on the grounds that it is an easily implemented method based on the familiar parsimony tree-building strategy. However, this method has one major disadvantage compared to the other supertree methods, in that parsimony is NP-complete (Graham and Foulds, 1982) and so supertree construction by this method may never be an efficient or speedy process.

The only apparent advantage of consensus methods over concatenation methods is the merely practical one that they can objectively combine any sources of data that can be represented as a tree (even subjective opinions). This leaves them open to abuse, of course, or at least extremely naïve use. Several potential disadvantages have been identified (partly summarized by Bininda-Emonds, 2004; Gatesy *et al.*, 2004; Wilkinson *et al.*, 2005), including: failure to include any source character information that was not included in the trees (e.g. there might be a slightly suboptimal pattern common to all of the trees that is not represented in any of the trees, which is called hidden support); ability to produce novel groups of taxa (possibly spurious) that do not occur in any of the source trees (this applies to all of the optimization supertree methods plus the Adams consensus, because these all require rooted input trees); pseudoreplication of the data (i.e. including the same information in several of the source trees, thus increasing its weight); sensitivity to both tree size and tree shape can bias the form of the supertree; it is inappropriate to apply most types of branch-support methods (e.g. bootstrapping); and the final trees are summaries of summaries (which makes them inefficient) that might also have no direct biological interpretation as phylogenies.

The latter problem is a common one when dealing with data summaries, and it is important to remember that most consensus methods are a tool for data representation rather than a tool for new phylogenetic inferences (Bryant, 2003).

8. WORKED EXAMPLE

To help make some of this theoretical background more concrete, it is important to consider a specific example of a phylogenetic analysis. For this purpose, I have chosen some molecular sequence data that will allow the construction of a set of gene trees for a set of taxa, and which can then be combined into a single species tree.

This example is simply an attempt to show some of the possibilities for phylogenetic data analysis, and to make some important points about the complexities, potentialities and pitfalls in the analysis of such experiments. I am not attempting an explicit re-analysis of this particular set of data, nor am I trying to provide a definitive analysis of the data, although you could do a lot worse than follow what I have done here. I will not be giving any biological interpretation of the results, although the obvious phylogenetic patterns will be highlighted. Furthermore, the data analyses discussed here can be carried out via a wide variety of computer programs. I have used a somewhat arbitrary selection of these, and my choice of these particular programs should not be seen as a recommendation. However, in science an explicit statement should always be made about which programs have been used, and which versions of those programs, just as the details of equipment and chemicals are provided for laboratory protocols.

If you want an alternative example, then Kjer (2004) and Rooney (2004) provide relatively recent analyses of large data sets for the 18S rRNA gene that exemplify the opposite extremes of phylogenetic analysis.

8.1. Taxa and Genes

For my example, I have chosen the molecular data discussed by Xiao *et al.* (2002), which they used to assess the evolution of *Cryptosporidium*

(Apicomplexa) parasites. These authors compiled sequence data for several genes, covering *Cryptosporidium* samples from a wide range of hosts. I will refer to these samples as “taxa” here, as the authors suggest that several of the genotypes should be recognized as separate species (and some have subsequently been named).

Unfortunately, there are many nomenclatural inconsistencies in the tables and figures presented by Xiao *et al.* (2002). Most of the taxa are represented by more than one *Cryptosporidium* isolate (see their Table 1), and some of these isolates are listed but apparently never used in the analyses, while others appear in the tables but not the figures and some appear in the figures but not the tables. I have therefore used only those taxa that I could unambiguously relate to a particular isolate and database sequence, by tracing the literature references provided. This choice will inevitably result in some stochastic error and possibly also some systematic error (which I would expect to resolve if I was an expert in this particular taxonomic group).

Extensive data are available in the DDBJ EMBL Genbank sequence databases for four *Cryptosporidium* genes: actin; 70 kDa heat shock protein (HSP70); small subunit ribosomal RNA (SSU); and cryptosporidium oocyst wall protein (COWP). However, the latter gene is restricted to *Cryptosporidium*, and so there can be no out-group to root the tree in a phylogenetic analysis. Therefore, for this exercise I have restricted myself to the partial sequences that are available for the first three of these genes. The final data sets contain 29, 22 and 32 taxa for the actin, HSP70 and SSU genes, respectively. This choice of multiple genes will help assess the degree of stochastic variation by allowing the repeatability between genes to be evaluated.

Note that I have no idea at all about the quality of these data, since I have obtained them entirely second hand. I am not the only one with a jaundiced view of much of the content of the sequence databases (e.g. Forster, 2003), based on hard-won experience, and whether we will ever do anything to raise the standards remains unknown (Bridge *et al.*, 2003; Harris, 2003). However, it is currently naïve to download data from a database without at least cross-checking them for internal consistency and also consistency with the original publication. Given that most of the public-access databases are not curated (i.e. the onus for accuracy of all of the details rests

entirely with the submitter), it is essential that all sequences be associated with properly vouchered specimens, if we expect to be able to verify the information at a later date. This simple criterion is apparently ignored by most of the people who submit sequences to these databases.

8.2. Outgroup

There is much ambiguity about the sister group to *Cryptosporidium* (Carreno *et al.*, 1999; Zhu *et al.*, 2000; Xiao *et al.*, 2002), which is necessarily the best choice as outgroup for a phylogenetic analysis. Therefore, I have simply followed precedent, and chosen a member from the coccidia (*T. gondii*), the piroplasms (*B. bovis*) and the haemosporidia (*P. falciparum*) to serve as the outgroup. These particular taxa were chosen solely because their actin, HSP70 and SSU genes have been sequenced and are publicly available. We can anticipate a considerable degree of systematic error as a result of these choices of outgroup.

As will be emphasized several times below, it turns out that the biggest problems in the data analysis all result from this outgroup. These taxa are evolutionarily too distant from *Cryptosporidium* to be particularly useful as an outgroup in a phylogenetic analysis. Clearly, some other members of the Apicomplexa are needed, although the choice will not be straightforward if *Cryptosporidium* is actually an early branching member of the Apicomplexa (e.g. Zhu *et al.*, 2000) remember, as outgroup we preferentially want to use basal members of the sister group. My first (and perhaps major) point is thus that members of a suitable outgroup need to be sequenced as part of any sequencing project if the data are to be used for phylogenetic purposes – you cannot rely on the fortuitous presence of the necessary outgroup sequences in a public database.

8.3. Sequence Alignment

The two protein-coding genes should be relatively straightforward to align automatically using a progressive-alignment computer program.

at least as far as the ingroup is concerned, as the average nucleotide identities among the taxa are 87% for both the actin and HSP70 genes. However, the average similarities of the ingroup sequences to the outgroup taxa are much less, being 71% and 69% respectively, which is getting near the lower end of reliability for programs such as ClustalW.

So, the actin and HSP70 gene sequences were aligned using the default parameters in ClustalW version 1.83 (Thompson *et al.*, 1994), and then checked by eye using MacClade version 4.05 (Maddison and Maddison, 2000). The only notable inaccuracy that needed manual editing was at the end of the HSP70 gene sequence, where there is a GGT GGT ATG CCA motif that is repeated > 8 times. The different ingroup taxa have minor variations on this motif that rarely change the amino acid coding, and so the automatic alignment algorithm was successful. However, the three outgroup taxa, while still recognizably retaining the motif, show much stronger variation—*T. gondii*, for example, is missing the CCA part in several of the repeats. The outgroup, thus needed considerable manual intervention in this region in order to align the motifs. It is perhaps also worth noting that both genes have locations in the alignment where several indels of <3 nucleotides occur in near succession, so that the amino acid translation changes in some taxa relative to the others over a short section of the sequence (i.e. a frame-shift)—this seems to be a real phenomenon rather than an artefact of the alignment algorithm, but frame-shifts of this nature can also be indicative of suspect database quality (Harris, 2003).

It has been repeatedly emphasized in the literature that rRNA genes are much more problematic to align than are proteins (Morrison and Ellis, 1997; Beebe *et al.*, 2000; Hickson *et al.*, 2000; Mugridge *et al.*, 2000; Kjer, 2004), as the highly conserved regions are interspersed with ultra-variable regions (usually single-stranded). The SSU sequences were therefore aligned using the rRNA secondary-structure information, by downloading the sequences in aligned form from the European Ribosomal Database (Wuyts *et al.*, 2004). The alignments were then extensively checked by eye to ensure consistency and congruence among taxa with similar sequences as such databases have not been manually curated and thus are not 100% reliable. The final

alignment used covers positions 218–1044 of the 1795 bp *T. gondii* SSU rRNA structure shown by Gagnon *et al.* (1996) due to the fact that many of the taxa have been only partially sequenced. The only problematic part of the alignment occurs in the hypervariable V4 region, covering helices E21-2 and E21-3. This region could possibly be deleted from the analysis; and, indeed, many people would probably insist that it should be deleted since it may not be reliable. However, for the purpose of this exercise I have chosen to retain it, so that the possible effects can be discussed. Note, also, that the use of structural information in the alignment will be needed for the subdivision of the alignment into functional partitions, as discussed below.

For all three genes, where there were multiple sequences of the same taxon (from different isolates) these were merged into a consensus sequence after alignment, using the standard IUPAC ambiguity codes for those nucleotide positions with more than one possible character state in the consensus sequence, as there were usually only minor differences among them. The only exception to this was for the two developmentally distinct actin gene loci of *P. falciparum* (Wesseling *et al.*, 1989), which were very different and were thus kept separate (labelled here as actin I and actin II), although the replicate sequences from each locus were merged. The two *P. falciparum* SSU sequences were merged, although they may also represent distinct developmental loci (McCutchan *et al.*, 1988), as they were quite similar; and the three *B. bovis* SSU sequences are not identical to each other in the hypervariable V4 region, suggesting that there may also be distinct developmental loci. The most divergent *T. gondii* SSU sequence (accession no. X65508) was not included in the data set, as its relationship to the other sequences is not clear (Rooney, 2004).

The beginning and/or end of some of the aligned sequences was then truncated to match those that were less completely sequenced, since different taxa were sequenced with different primers. The final alignments contained 1006, 1880 and 907 positions for the actin, HSP70 and SSU genes, respectively. These alignments have been deposited in both the EMBL-Align (Lombard *et al.*, 2002) and TreeBASE (Morell, 1996) public-access sequence-alignment databases.

under the accession numbers ALIGN_000951, ALIGN_000956 and ALIGN_000960 (EMBL-Align) and S1404 (TreeBASE), respectively. The complete files, including the aligned data, the program instructions for performing the final analyses described below and the resulting phylogenetic trees, are also available at <http://hem.fyristorg.com/acacia/alignments.htm>.

8.4. Model Assessment

This is clearly a complex business, being related to the overall problem of systematic error, and so I have tried to be thorough for this example. For the purposes of this exercise, I considered the following aspects of the model for each gene: (i) quantifying all six components of the phylogenetic model specification shown in Figure 5; (ii) partitioning the different sites into functional groups to assess possible incongruences within the genes; and (iii) assessing the effect of the outgroup on the estimates of ingroup relationships. These aspects are all inter-related, and so they are discussed together here. However, some of the tests actually need to be performed before the tree-building analyses are run, in order to decide on the appropriate details of the analysis, while others are performed after the tree-building analyses are run, in order to work out which parts of the results are unreliable.

For the partitioning of nucleotide sites into functional groups, I examined only the most commonly used groupings from the literature. For the two protein-coding genes this involved the three codon positions, separately or in combination, while for the SSU gene it involved the single-stranded (e.g. loop) and double-stranded (e.g. helix) positions. The literature suggests that the protein 3rd-codon positions, for example, might be different from the other positions, while the single-stranded rDNA regions contain most of the gapped alignment (and thus any alignment ambiguity). The codon positions were determined by using MacClade to find the only reading frame for each gene that did not have multiple stop codons, while the single- and double-stranded positions were determined with reference to the *T. gondii* SSU rRNA structure shown by Gagnon *et al.* (1996).

Phylogenetic analyses were then performed for each partition separately as well as for the gene sequence as a whole. To test the effect of the outgroup, the analyses were performed separately with and without the outgroup taxa. The tests used for the model specifications are listed in Figure 8.

The tests for the stationary models (the top row of Figure 8) were all conducted together. The hierarchical likelihood-ratio tests and Akaike information criterion were carried out using Modeltest version 3.06 (Posada and Crandall, 1998) and PAUP* version 4.0b10 (Swofford, 2002), while the decision theory tests were carried out

	Base frequencies $P_A = P_C = P_G = P_T$	Nucleotide substitutions $A \leftrightarrow C = A \leftrightarrow G = A \leftrightarrow T =$ $C \leftrightarrow G = C \leftrightarrow T = G \leftrightarrow T$	Among-site rate variation $I = 0; \alpha = \infty$
Stationary models	(a)	(b)	(c)
Tests used:	likelihood-ratio test / AIC / decision theory	likelihood-ratio tests / AIC / decision theory for combinations of ti and tv	likelihood-ratio tests / AIC / decision theory for (i) $\alpha = \infty$; (ii) $I = 0$
Test results:	rejected for all genes and subsets	almost always rejected except for sometimes $ti_1 = ti_2$	(i) rejected (ii) accepted for actin, rejected for HSP and SSU
Non-stationary models	(d)	(e)	(f)
Tests used:	(i) overall χ^2 (ii) individual χ^2	(i) symmetry χ^2 (ii) heuristic χ^2	(i) Huelsenbeck test (ii) Lockhart-Steel test
Test results:	(i) accepted for SSU but rejected for actin and HSP due to 3rd codon positions (ii) rejected for outgroup and some actin and HSP	(i) accepted for ingroup for SSU but rejected for actin and HSP mainly due to 3rd codon positions (ii) accepted for ingroup	(i) accepted for actin but rejected for HSP and SSU (ii) rejected between outgroup and ingroup but accepted within ingroup

Figure 8 The six different components of an evolutionary model that can potentially affect the results of a phylogenetic analysis (from Figure 5), along with the results of performing some of the statistical tests for the phylogenetic analysis of the *Cryptosporidium* data. The final analysis was performed by partitioning each of the three genes into two subsets and then allowing separate GTR + I + Γ models for each of these six partitions. This was because the statistical tests indicated that the assumption of constancy was violated for (a) + (b) + (c) but not (f), as shown in the appropriate cells of the figure. Some non-constancy of (d) and (e) was detected within a few of the partitions, and this may have an unknown effect on the results of the analysis.

using DT-ModSel (Minin *et al.*, 2003) and PAUP*. These three tests take the same general approach to testing the models, by constructing a base tree and then varying the details of each of the model components in all combinations for that tree, in order to assess the 56 possible models shown in Table 1. That is, the methods all standardize the indel model and tree model, and then vary the nucleotide-substitution model. However, the tests differ in the criterion that they use to decide which of the 56 models is best. It is probably unnecessary to perform all three of these tests—I have done so merely for the purposes of this exercise.

For the *Cryptosporidium* data, I performed the tests separately for each of the three genes and separately for each of the functional partitions (with the outgroup included in all cases), as shown in Table 2. The three tests are in basic agreement with each other within each of the 13 sets of analyses performed, which is reassuring, although they sometimes differ in details. For example, the tests always agree concerning the assessment of base frequencies and almost always agree on the assessment of among-site rate variation, with most of the disagreements being about the nucleotide substitutions.

For the analyses of the complete gene sequences, all of the tests agree that the assumption of equal base frequencies is unjustified for these data, and also that among-site rate variation needs to be taken into account using a gamma distribution. Furthermore, the HSP70 and SSU genes, but not the actin gene, appear to have a large proportion of invariable sites that should be taken into account in the model. Finally, for all three genes there seems to be a complex relationship among the rates of nucleotide substitutions, so that complex models are needed in which all (or almost all) of the substitution rates are unequal. In other words, all of the simpler models are rejected as inadequate, and the most appropriate mathematical model for the phylogenetic analysis must allow the base frequencies to vary, all six substitution rates to vary (i.e. the general time-reversible substitution model, GTR), a proportion of the sites to be invariant (for two of the three genes), and the variable sites to vary with a gamma distribution—this is referred to here as the GTR+I+ Γ model (it is also known in the literature as a “rates across sites” model).

Table 2 Substitution models chosen as a result of the likelihood-ratio test, Akaike information criterion and decision theory test for each of the genes and partitions investigated

Partition	Gene		
	Actin	HSP70	SSU
All sites	GTR+Γ ^a	GTR+I+Γ	GTR+I+Γ
	GTR+Γ ^b	GTR+I+Γ	TVM+I+Γ
	TVM+Γ ^c	GTR+I+Γ	TVM+I+Γ
1st codon	TrN+Γ	TrN+I+Γ	—
	GTR+Γ	GTR+I+Γ	
2nd codon	GTR+Γ	GTR+Γ	
	F81+Γ	F81+Γ	—
	GTR+Γ	GTR+I	
1st+2nd codon	JC+Γ	TVM+I	
	TrN+Γ	TrN+I+Γ	—
	GTR+I+Γ	GTR+I+Γ	
3rd codon	GTR+Γ	GTR+Γ	
	GTR+I+Γ	TVM+I+Γ	—
	TVM+I+Γ	TVM+I+Γ	
Single-stranded	—	—	GTR+I+Γ
			TVM+I+Γ
			K81uf+I+Γ
Double-stranded	—	—	TVM+I+Γ
			TVM+I+Γ
			TVM+I+Γ

^aLikelihood-ratio test.
^bAkaike information criterion.
^cDecision theory test.

However, the situation is actually slightly more complex than this, because it is also clear from Table 2 that the different functional partitions differ from each other in the required model. Notably, for the two protein-coding genes the model chosen for the 3rd-codon positions is more complex than that chosen for the 1st and 2nd positions. In particular, the tests suggest that a proportion of invariant sites is needed in the model for the actin 3rd-codon positions but not for the 1st or 2nd positions. Such a conclusion is not unexpected, as it is universally recognized that in protein-coding genes the 3rd-codon positions have a greater evolutionary rate, since substitutions often do not affect the translated amino acid sequence. Less obviously, the

single- and double-stranded regions of the SSU gene also seem to have different evolutionary models. Once again, this is not unexpected, as the double-stranded sites have less substitutional freedom due to the necessity of base pairing.

At this stage it is worth noting, as an aside, that the model testing done here is somewhat limited, in the sense that the Modeltest and DT-ModSel programs only test single-nucleotide models of evolution (see Section 5.1). It is entirely possible that a doublet model may be more appropriate for the SSU gene sequence, for example, and that a codon model may be more appropriate for the two protein-coding genes (see Section 5.4). I have not explored these more-complex possibilities here, but I might do so if I were to analyze these data as part of my own research project.

Having decided on the “best” model to use, it is then possible to perform the tree-building analyses. For this I employed Bayesian analysis, using MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003). I made this choice solely on the grounds that the complex substitution models I was investigating are best addressed in a likelihood context, and large numbers of maximum-likelihood analyses are impractical –several preliminary analyses that I ran for a single gene were still incomplete even after a week of computer time.

Default values were used for all of the Bayesian analyses unless specified otherwise. My preliminary analyses indicated that the likelihood parameters converged after 10 000 generations, and so this was used as the burn-in period, followed by 1 000 000 further generations that were sampled every 100 generations, to produce 10 000 trees. All of the results reported here are based on the posterior probabilities derived from these 10 000 sampled trees. Several random starts were tried for the preliminary analyses, to ensure that the Markov chain had converged, and graphs of the various model parameters were examined to ensure that the Markov chain was mixing well. No problems were found, with the exception of the example shown in Figure 6.

I performed the Bayesian analyses separately for each gene and for each functional partition within each gene. The GTR + I + Γ model has 10 parameters for which the values need to be estimated as part of each tree-building analysis. The values of these model parameters

Table 3 Mean parameter values estimated by Bayesian analysis using the GTR + I + Γ model, as applied to each of the three genes (including out-group) and separately to each of their two partition subsets

Parameter	Actin			HSP70			SSU		
	Overall	1st + 2nd ^a	3rd	Overall	1st + 2nd	3rd	Overall	Single	Double
$r(G \leftrightarrow T)^b$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
$r(C \leftrightarrow T)$	61.395	16.392	69.763	62.390	10.066	67.099*	4.878	3.627	8.354
$r(C \leftrightarrow G)$	6.551	4.163	1.524	3.993	2.307	5.610	0.291	0.419	0.316
$r(A \leftrightarrow T)$	20.440	3.435	20.427*	19.964	2.007	34.736*	4.140	2.447	7.234
$r(A \leftrightarrow G)$	51.755	3.261	70.313*	32.449	2.744	71.628*	4.230	3.416	6.853
$r(A \leftrightarrow C)$	9.223	6.689	4.166	9.005	4.593	6.575	1.101	1.119	1.562
$\pi(A)^c$	0.296	0.304	0.306	0.293	0.338	0.243*	0.288	0.329	0.254*
$\pi(C)$	0.187	0.205	0.153*	0.233	0.206	0.253*	0.169	0.149	0.182
$\pi(G)$	0.202	0.253	0.163*	0.236	0.267	0.194*	0.211	0.169	0.245*
$\pi(T)$	0.315	0.238	0.378*	0.238	0.189	0.310*	0.331	0.353	0.319
α^d	0.306	0.563	3.350*	0.886	2.945	5.096	0.312	0.282	0.397
$p\text{-invar}^e$	0.047	0.208	0.036	0.350	0.474	0.024*	0.282	0.161	0.321

^a1st + 2nd, first- and second-codon positions; 3rd, third-codon positions; Single, single-stranded positions; Double, double-stranded positions.

^bRate of substitution between the specified bases relative to the G \leftrightarrow T rate.

^cProportion of the specified base.

^dShape parameter for the among-site rate variation.

^eProportion of invariable sites.

*95% credible intervals for the two partitions do not overlap.

resulting from each of the Bayesian analyses (including outgroup) are shown in Table 3. The values can be compared between analyses using the 95% credible intervals, which are an analogue of 95% confidence intervals—if two credible intervals do not overlap then the parameter values can be considered to be statistically significantly different from each other.

Several conclusions can be reached from these parameter data. First, the parameter values for the three genes are quite different from each other. This will be important when we come to consider constructing the species tree from the three gene trees—clearly, we cannot just pool the three data sets but must instead consider how best to combine the three heterogeneous sources of information. Second, the parameter values for the different functional partitions within each gene are quite different from each other, notably for the two protein-coding genes. In particular, the 3rd-codon positions for both protein-coding genes have a lower proportion of invariant sites and a higher

rate of $C \leftrightarrow T$ and $A \leftrightarrow G$ substitutions, both of which are expected consequences if the data are becoming saturated by multiple substitutions (Larson, 1994; Cunningham, 1997a). This potential problem can be checked by performing a saturation analysis, as shown in Figure 9. This analysis confirms that the 3rd-codon positions are indeed becoming saturated while the 1st + 2nd positions are not, to an extent that can affect the tree-building analyses.

These observations confirm the suggestions from the model tests, in that there are differences between the functional partitions that will affect the tree-building analyses. This will also be important when we come to construct the gene trees—clearly, we should keep the functional partitions separate in the tree-building analyses even if we are using the same model for each partition and gene. Forcing all of the sites into the same set of model parameters will only lead to mis-estimation of the phylogenetic tree, which may involve the branching order and/or the branch lengths.

Having decided on suitable partitions and substitution models, we are now ready to produce the three gene trees. I did this by applying the GTR + I + Γ model separately to each gene while allowing the model parameters to vary between the two functional partitions within each gene. The gene trees are shown in Figure 10. Each of these is a Bayesian posterior probability tree, which is a majority-rule consensus tree from the 10 000 trees sampled by the Bayesian analysis. The clade-credibility values from the analyses, which indicate the percentage of the 10 000 sampled trees that contained each of the branches, are also shown.

When interpreting these gene trees we need to take into account two factors: the differences between the trees produced by the functional partitions independently, and the effect of the outgroup taxa (which root the tree). These effects can be assessed by performing the tree-building analyses both with and without the outgroup taxa.

The first indication of differences between the two functional partitions for each gene is in how structured the trees are. This can be assessed by the treeness statistic of Lanyon (1988), which quantifies the proportion of the branch lengths that are attributable to the internal branches (which determine the topology of the tree) as opposed to the external branches. For the two protein-coding genes the

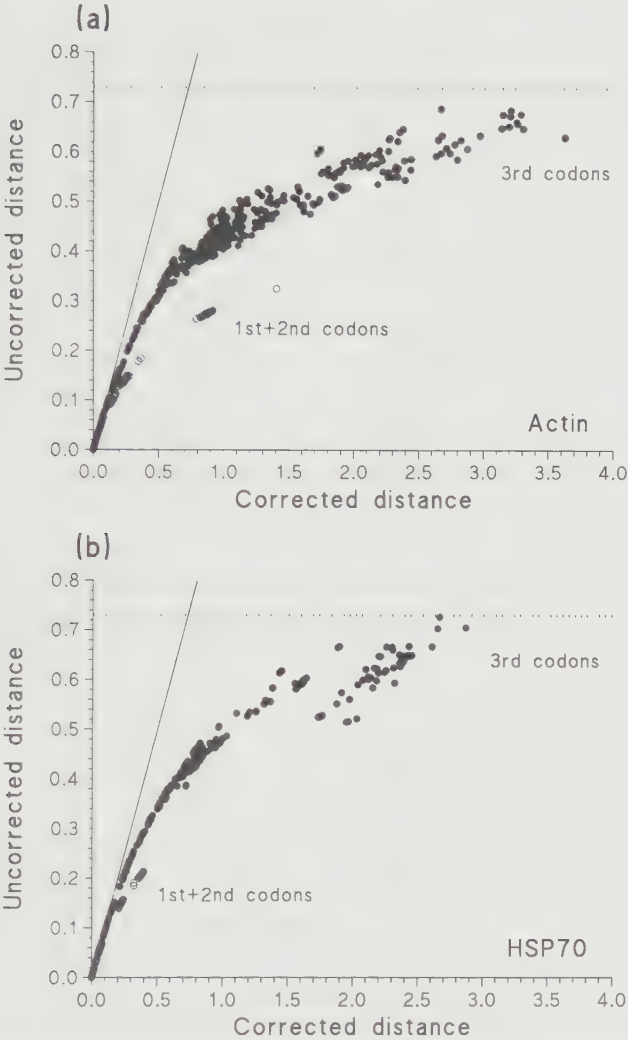
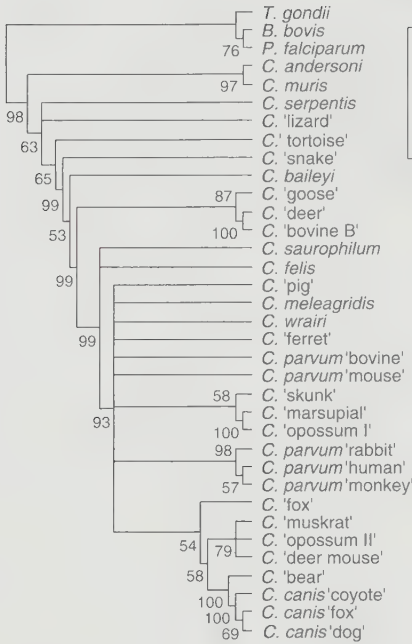
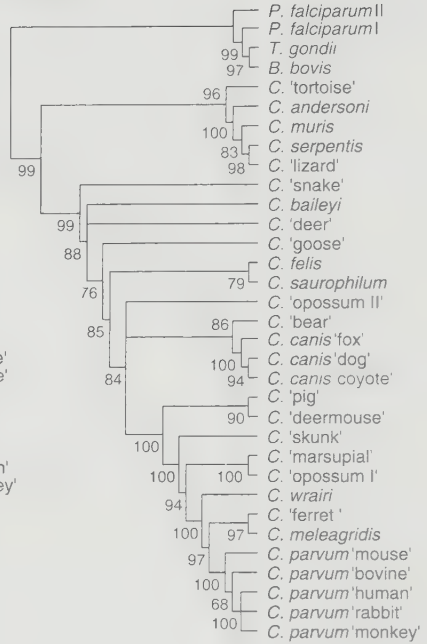


Figure 9 Saturation analysis of the 3rd-codon position (solid circles) and 1st + 2nd-codon positions (open circles) for the (a) Actin and (b) HSP70 genes of the *Cryptosporidium* taxa (including outgroup). Each point represents a pairwise comparison among the taxa, with the uncorrected distance being the observed proportion of differences between taxa and the corrected distance being the expected number of differences based on the substitution models shown in Table 3 (estimated via maximum likelihood using the PAUP* version 4.0b10 program). For both genes, the 3rd-codon positions are approaching saturation by multiple substitutions (shown as the dotted line), whereas the 1st + 2nd-codon positions are not. Beyond about 30–40% observed sequence divergence the number of multiple substitutions can become problematic, and serious loss of phylogenetic signal will ensue.

SSU



Actin



HSP70

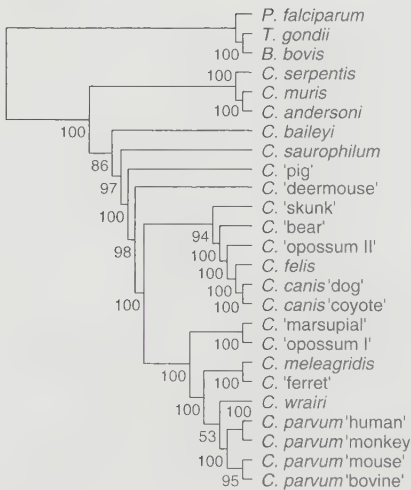


Figure 10 Majority-rule consensus tree based on the Bayesian posterior probability for the *Cryptosporidium* taxa for each of the three genes ("gene trees"). The clade-credibility (or posterior-probability) values are shown for each branch of the tree, which indicate the percentage of the sampled trees containing that branch. The data were analyzed for each gene using the GTR + I + Γ substitution model, based on two functional partitions.

1st + 2nd codon partition produces a slightly more structured tree than does the 3rd-codon partition (actin: 0.354 versus 0.311; HSP70: 0.584 versus 0.514), and for the SSU gene the single-stranded partition is slightly more structured than is the double-stranded partition (0.431 versus 0.422).

As far as the outgroup is concerned, there are two observable effects on the results of the tree-building analyses. First, when the outgroup is excluded then several of the polychotomies shown in Figure 10 are resolved. That is, including the outgroup taxa reduces the ingroup resolution of the gene trees – when the outgroup is excluded there are five extra branches on the SSU gene tree, two on the actin tree and one on the HSP70 tree. However, the topology of the trees is otherwise maintained, which is reassuring. Second, the position of the outgroup differs for different functional partitions. For both of the protein-coding genes, the 1st + 2nd codon partition roots the tree as shown in Figure 10, while the 3rd-codon partition places *P. falciparum* as shown in Figure 10 but places *T. gondii* + *B. bovis* as the sister to the *C. canis* group of taxa (for the actin gene) or to *C. felis* (HSP70). Furthermore, for the SSU gene the double-stranded partition roots the tree as shown in Figure 10, but the single-stranded partition roots the tree so that *C. serpentis* and *C. "lizard"* are the sister to *C. andersoni* + *C. muris* (the ambiguity of the root placement here is indicated by the low clade-credibility values of 63% and 65%). That is, the actin and HSP70 1st + 2nd-codon partitions plus the SSU single-stranded partition all have approximately the same root to the gene tree, while the other three partitions at least partially disagree. The outgroup is thus clearly problematic.

The source of this problem can be investigated by looking at the assumption of stationarity of the models (the bottom row of Figure 8). This is currently an *ad hoc* affair, as there are all sorts of tests available and no coherent framework for employing them, as discussed in Section 5.2.

We can start by looking at the base frequencies (the first column of Figure 8). Each sequence can be tested individually by comparing its nucleotide composition to that expected under the evolutionary model using a goodness-of-fit χ^2 test, via the Tree-Puzzle version 5.0 program (Strimmer and von Haeseler, 1996). Applying these tests to

each gene immediately rejects the outgroup taxa as not fitting the model. That is, the outgroup taxa do not have the same base composition as the ingroup taxa, which violates one part of the evolutionary model that was used to construct the gene trees. This may help to explain why the rooting of the tree is unstable.

These analyses also highlight several other taxa as having unusual base compositions within the ingroup. For example, both the actin and HSP70 genes show biased composition for *C. felis*, *C. "opossum II," C. canis "dog"* and *C. canis "coyote,"* and the actin gene also shows it for *C. canis "fox"*. Note that these taxa tend to group together on the gene trees, which at least raises the possibility that the grouping might represent an artefact of violating the model. However, this suggestion is vitiated by the same taxa grouping together on the SSU tree as well, where there is less compositional bias. This compositional bias thus probably represents a real evolutionary trend (i.e. homology rather than analogy).

The compositional stationarity of the ingroup can be further investigated in relation to the functional partitions via a contingency χ^2 test, using PAUP*. Two sets of analyses are shown in Table 4, both with and without the constant sites (as explained in Section 5.1)—the "true" answer probably lies somewhere between the two analyses. The analyses confirm that there is compositional non-stationarity in both of the protein-coding genes, and suggest that it might also occur in the SSU gene. More importantly, the analyses attribute the compositional biases solely to the 3rd-codon partition for both protein genes, and not to the 1st + 2nd partition. This means that the analysis of the former partition may not be reliable, and may help to explain why the rooting of the tree differs between the two partitions.

Next, we can consider the differences between lineages in among-site rate variation (the third column of Figure 8)—this has recently been shown to be a necessary component in phylogenetic models for both the SSU (Lockhart *et al.*, 1998; Morrison *et al.*, 2004) and HSP70 (Germot and Philippe, 1999) gene sequences. We can start this by using the approximate likelihood-ratio test of Huelsenbeck (2002), which tests the overall phylogeny. This is done by performing the tree-building analyses with and without an allowance for a covarion-like component in the model. The results of these analyses are

Table 4 Results for each of the three genes (excluding outgroup) of the contingency χ^2 test for among-taxa equality of base composition, the Waddell Steel χ^2 test for reversibility of base substitutions and the heuristic χ^2 test for stationarity of base substitutions in the GTR + I + Γ evolutionary model

Gene Partition ^a	Base composition ^b		Base composition ^c		Reversibility		Stationarity ^d	
	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Actin	112.52	0.021	355.19	<0.001	161.22	<0.001	9.41	0.152
1st + 2nd	2.96	>0.999	25.03	>0.999	14.44	0.025		
3rd	405.12	<0.001	461.10	<0.001	74.30	<0.001		
HSP70	384.20	<0.001	1042.73	<0.001	205.02	<0.001	5.49	0.483
1st + 2nd	8.15	>0.999	74.55	0.151	16.55	0.011		
3rd	1088.75	<0.001	1198.06	<0.001	197.14	<0.001		
SSU	115.78	0.166	278.18	<0.001	11.02	0.088	0.86	0.990
Single			134.07	0.003				
Double			278.18	<0.001				

^a1st + 2nd, first- and second-codon positions combined; 3rd, third-codon positions; Single, single-stranded regions; Double, double-stranded regions.

^bIncluding all sites.

^cExcluding constant sites.

^dTesting *C. andersoni* + *C. muris* + *C. "lizard"* + *C. serpentis* + *C. "tortoise"* versus the remaining taxa.

shown in Table 5, which rejects the stationary model for both the HSP70 and SSU genes. This apparent problem can be further investigated using the inequality test of Lockhart *et al.* (1998) and Steel *et al.* (2000b), directly comparing the ingroup to the outgroup. This test makes it clear that the outgroup once again is causing a problem (Table 5) the among-site rate variation is not the same in the outgroup as in the ingroup. This may also help to explain why the rooting of the tree is unstable.

To investigate any non-stationarity within the ingroup itself, we need to define some subsets of ingroup taxa. The "majority opinion" from the rooting of the gene-tree analyses seems to be that *Cryptosporidium* contains two main monophyletic groups: (i) *C. andersoni* + *C. muris* + *C. "lizard"* + *C. serpentis* + *C. "tortoise"* (note that *C. "lizard"* and *C. "tortoise"* have not been sequenced for the actin gene), and (ii) the rest of the taxa. This is indicated by the relatively large clade-credibility values for group (ii) in all three genes

Table 5 Results for each of the three genes of the various tests of the covariotide evolutionary models: the Huelsenbeck approximate likelihood-ratio test for the overall comparison of equal evolutionary rates among lineages, and the two Lockhart–Steel tests for equal evolutionary rates among lineages for the two taxon subsets

Gene	Approximate likelihood		Ingroup versus outgroup		Ingroup subsets ^a	
	δ	P	z	P	z	P
Actin	3.24	0.109	4.11	>0.999	2.84	0.998
HSP70	5.42	0.036	-1.74	0.041	-0.78	0.218
SSU	100.80	<0.001	-3.48	<0.001	0.78	0.782

^aTesting *C. andersoni* + *C. muris* + *C. “lizard”* + *C. serpentis* + *C. “tortoise”* versus the remaining taxa.

(99%, 99% and 86%; see Figure 10) and the large clade-credibility values for group (i) in the actin and HSP70 genes (96% and 100%).

The results of applying the Lockhart–Steel test to these two subsets of taxa is shown in Table 5, indicating that there is no detectable violation of stationarity of the among-site rate variation. This is reassuring, as it restricts any potential problems to the outgroup relationship.

Finally, we can check the stationarity of nucleotide substitutions (the second column of Figure 8). I tested reversibility of the nucleotide-substitution model (excluding the outgroup) using the symmetry goodness-of-fit χ^2 test (Waddell and Steel, 1997), based on the final gene trees and the average number of nucleotide substitutions inferred using MacClade. The results of these analyses (Table 4) are very similar to those for the assessment of base composition. That is, the analyses indicate that there is substitution non-stationarity in both of the protein-coding genes, and this is attributable solely to the 3rd-codon partition for both genes and not to the 1st + 2nd partition. This may also help to explain why the rooting of the tree differs between the two partitions.

I also examined stationarity of the nucleotide-substitution model using a heuristic goodness-of-fit χ^2 test, comparing the observed number of substitutions for the two subsets of ingroup taxa with the expected values for the whole data set, based on the final evolutionary

tree and the average number of nucleotide substitutions inferred using MacClade. No differences between the two groups were detected (Table 4). However, this is only a rough test because the parsimony count will be an underestimate of the true number of substitutions.

8.5. Tree-Building Analysis for the Species Tree

Having thus assessed the data and found an evolutionary model that might fit it (different model parameters estimated for two partitions for each of the three genes), as well as assessing the extent to which the model might not fit it (potential problems with the outgroup due to non-stationarity), we can proceed to build a species tree from the gene trees. Here, I explore the two approaches to this problem that are available when not all of the taxa appear in all of the component data sets.

First, we can pursue the logical line that was followed to arrive at the gene trees. That is, we can build a single tree from the combined data using a specified evolutionary model. In this case, the model is straightforward to derive from the models used for the gene trees. The most appropriate model is the $GTR + I + \Gamma$ model, allowing the model parameters to vary between the three genes and the two functional partitions within each gene (i.e. a total of six partitions). This use of six partitions is necessary because we already know that the genes as well as their functional partitions have detectable differences that affect the resulting tree, so we need to allow each of these partitions to have its own values for all of the substitution parameters. We also need to constrain the analysis so that the branch lengths are proportional across the different data partitions but the same tree topology is used for all of the partitions, so that we are searching for the tree that is common across all of the genes (which should be the species tree). Thus, we have a combined model in which the substitution-model parameters are independent across the partitions while the tree-model parameters are dependent across the partitions. Furthermore, we also expect that this combined model will be violated to some extent, in the sense that there will be some non-stationary aspects, and that the root might not be stable (i.e. we have expectations

from the single-gene analyses as to the systematic error we might encounter).

An alternative strategy to deal with these problems might be, for example, to remove the 3rd-codon positions from the analysis. I do not usually advocate deleting data, but instead consider it preferable to provide a separate partition for those data, although many people will disagree. Similarly, the *C. "fox"* sequence could be deleted from the species tree because it has only been sequenced for one of the three genes, and it may therefore be problematic as a result of lack of phylogenetic information. For this exercise, I have chosen to proceed with the full data, and will note the potential problems as I proceed.

So, I once again analyzed the data via MrBayes. A preliminary analysis indicated that the likelihood parameters converged after 40 000 generations, and so this was used as the burn-in period, followed by 1 000 000 further iterations that were sampled every 100 iterations to produce 10 000 trees. The final Bayesian posterior probability tree is shown in Figure 11, this being the majority-rule consensus tree from the 10 000 trees sampled by the Bayesian analysis. The clade-credibility values from the analyses, which indicate the percentage of the 10 000 sampled trees that contained each of the branches, are also shown, as are the estimated branch lengths indicating the amount of evolutionary change along each branch. The position of the root is indicated, but the outgroup taxa themselves are not shown.

As before, the tree needs to be evaluated in terms of the contributions from the component partitions. The position of the root is the "majority opinion" root referred to above, and it is supported by 100% clade-credibility scores. This means that the instability apparently caused by the model violations due to the outgroup taxa has been overcome. Furthermore, all of the small clade-credibility values (I am interpreting "small" as $<90\%$) can be directly attributed to known conflicts among the three gene trees, remembering that ambiguous placement of even a single taxon will affect several branch-support values due to non-independence of the branches (see Section 3.3). For example, the values of 57%, 67% and 87% in the *C. "deer-mouse"* + *C. "pig"* + *C. "muskrat"* + *C. "fox"* group are due to the fact that: (i) the *C. "fox"* taxon has only been sequenced for the SSU

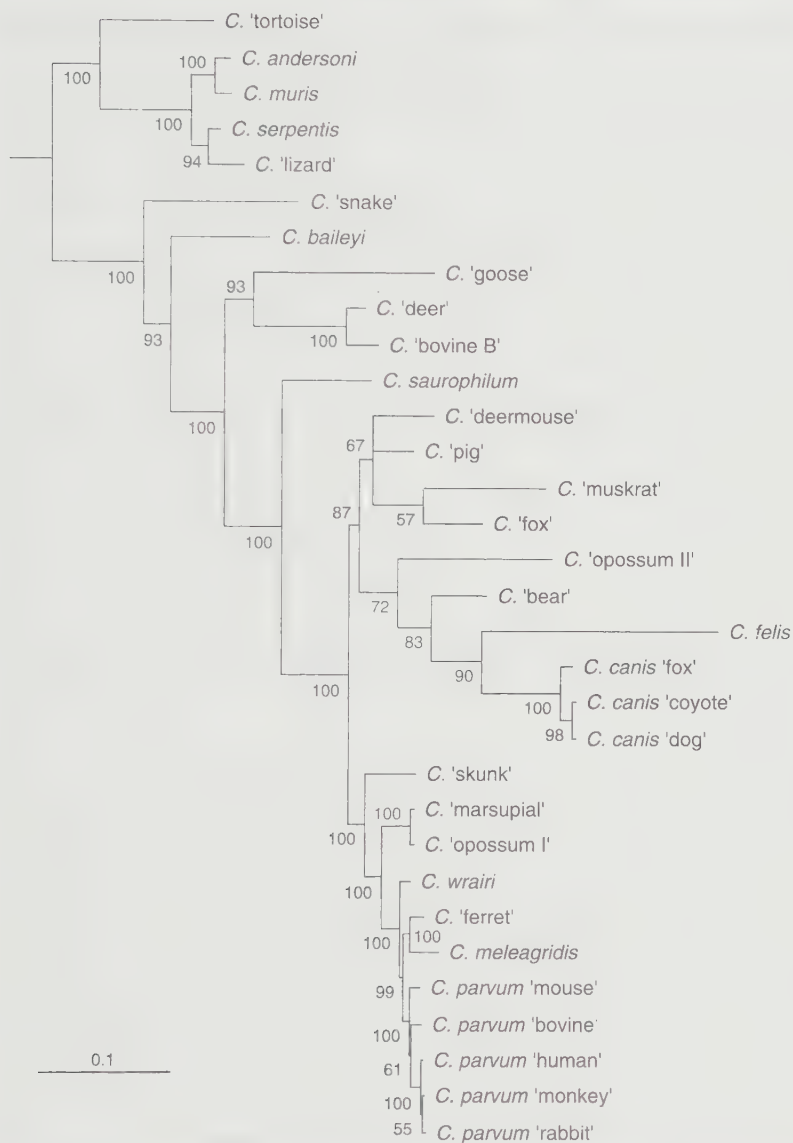


Figure 11 Inferred phylogenetic tree for the *Cryptosporidium* taxa ("species tree"), based on Bayesian maximum posterior probability. The branch lengths indicate the inferred amount of evolutionary change, according to the scale bar shown, with the outgroup taxa (*B. bovis*, *P. falciparum*, *T. gondii*) excluded due to their extremely long branch lengths. Also shown are the clade-credibility (or posterior-probability) values for each branch of the tree, which indicate the percentage of the sampled trees containing that branch. The data were analyzed using a separate GTR + I + Γ substitution model for each of the six data partitions.

gene, which contains very little information about the placement of this taxon, and (ii) only the actin 3rd-codon partition and the SSU double-stranded partition place *C. "pig"* in this position—it is placed with the *C. "skunk"* group by the SSU single-stranded partition and the actin 1st + 2nd-codon partition, and as a basal taxon by the two HSP70 partitions. Similarly, the values of 72%, 83%, 87% and 90% in the *C. "opossum II"* + *C. "bear"* + *C. felis* + *C. canis* group are due to the fact that only the HSP70 3rd-codon partition places *C. felis* in this position—it is placed with *C. saurophilum* by the actin 3rd-codon partition and the HSP70 1st + 2nd-codon partition, with *C. "pig"* by the actin 1st + 2nd-codon partition, and as a basal taxon by the two SSU partitions. As noted above, it may actually be the biased base composition that places *C. felis* where it is in Figure 11. The clade-credibility values of 55% and 61% in the *C. parvum* group represent groupings that are poorly supported by all of the gene partitions.

An alternative approach to constructing a species tree for these taxa is to use a supertree method. That is, we try to combine the topology information from the individual gene trees to produce a consensus species tree, even though not all of the taxa appear in all of the gene trees. In this case, all of the caveats about the individual trees apply, as discussed above—any inadequacies in the gene trees will be directly transferred to the species tree either as incompatibilities or as lack of information. For the analysis, I used the modified mincut supertree method of Page (2002), which is basically a supertree version of Adams consensus, as implemented in the Rainbow version 1.3 program (Chen *et al.*, 2004).

The topology of the resulting tree is very similar to the tree shown in Figure 11 (this sort of supertree does not have quantitative branch lengths, however). The differences all relate to points raised above. First, the root of the tree is shifted slightly, so that *C. "tortoise"* is not included in the group with *C. andersoni* + *C. muris* + *C. "lizard"* + *C. serpentis*. Second, *C. felis* is moved basally, to be just inside *C. saurophilum*. Third, *C. "fox"* and *C. "pig"* are moved sideways to be the paraphyletic sisters to the *C. "skunk"* group. Finally, *C. parvum* "human" + "monkey" + "rabbit" form a trichotomy. It needs to be borne in mind, when considering these differences, that the supertree is a summary tree that may or may not be a representation of a real

phylogenetic tree. Note, also, that this might be the first published direct comparison of a partitioned likelihood analysis with a supertree analysis – all previously published comparisons seem to have been between a single-partition parsimony analysis and a supertree analysis (usually MRP). Therefore, the strong congruence between my two analyses is quite interesting, in a general sense.

In conclusion, the detailed evaluation shows that the three genes seem to contain a strong and consistent phylogenetic signal for most of the taxa, and are ambiguous about the placement of only three of the 32 ingroup taxa. The placement of the root is somewhat uncertain, due to the fact that the outgroup taxa are evolutionarily too distant from the ingroup, so that the evolutionary processes are not constant across a tree that includes both the ingroup and the outgroup. The final species tree can now be interpreted in terms of the biological questions originally posed.

9. SUMMARY PROTOCOL FOR MOLECULAR SEQUENCES

If you wish to build a species tree from a set of molecular data, as I have done in the previous section, then the appropriate steps are as listed below. Exploring the characteristics of the data is seen as being more important than merely building a tree, since the tree will only be as good as the analysis makes it. This is the only way to avoid systematic errors and to minimize stochastic errors. Systematic errors are assessed by comparing different analyses, while stochastic errors are assessed by quantifying branch support.

- (i) Decide on the sample of taxa that will serve as the representatives of the ingroup and the genes that will serve as the representatives of the genome. You will need several taxa that represent each of the expected clades within the ingroup, particularly the expected basal taxa. You will need multiple genes if you want to have a reasonable estimate of the species tree, as these are the best way of dealing with many of the main sources of systematic variation.

- (ii) Decide on the outgroup taxa. This group needs to have several representative taxa from the sister group to the ingroup, preferably basal members. The more distant the evolutionary relationships to the outgroup then the less likely it is that the same evolutionary model can be fitted to both the ingroup and the outgroup, and the longer will be the connecting branches so that the root of the tree will be effectively random.
- (iii) Align the sequences. If the sequences have $<80\%$ nucleotide identity then automatic progressive alignment will be unreliable, and alternatives will be needed. Alternatives include the use of more sophisticated programs and the use of structural information. The final alignment should always be checked carefully by eye, and if there is any serious doubt then exclusion or down weighting of some regions can be considered.
- (iv) Choose a substitution model. This can be done relatively automatically these days for stationary single-nucleotide or amino acid models, if you have the right computer programs. Choosing and implementing a more realistic model is currently a more complex exercise.
- (v) Build a gene tree for each gene. This may involve a different model for each gene.
- (vi) Assess the possible functional partitions for evidence of different evolutionary processes. For proteins, 3rd-codon positions are often different from the others, as are the single- and double-stranded regions of rRNA.
- (vii) Assess the effect of the outgroup on the ingroup topology. Perform the tree-building both with and without the outgroup, to see if the root placement is stable, and if there is any effect on the ingroup relationships.
- (viii) Check for possible non-stationarity of the models. This can include base composition, substitutions and rates-across-sites. This may help to explain instabilities or odd groupings on the tree. Unfortunately, this is currently an *ad hoc* affair, with neither a simple protocol nor an automated program.
- (ix) Build the species tree, based on the best model as determined from the results of the previous steps. This may involve using a partitioned analysis if the previous steps indicate heterogeneity

in the combined data. Alternatively, a supertree can be constructed.

- (x) Interpret the tree as best you can, taking into account the limitations arising from your careful analysis (i.e. assessment of both stochastic and systematic error). Consider using a more sophisticated model if your model assessment indicates really serious heterogeneity in the data, or your final tree is biologically unbelievable.

REFERENCES

- Abdo, Z., Mimin, V.N., Joyce, P. and Sullivan, J. (2004). Accounting for uncertainty in the tree topology has little effect on the decision-theoretic approach to model selection in phylogeny estimation. *Molecular Biology and Evolution* **22**, 691–703.
- Addario-Berry, L., Chor, B., Hallett, M., Lagergren, J., Panconesi, A. and Wareham, T. (2004). Ancestral maximum likelihood of phylogenetic trees is hard. *Journal of Bioinformatics and Computational Biology* **2**, 257–271.
- Adell, J.C. and Dopazo, J. (1994). Monte Carlo simulation in phylogenies: an application to test the constancy of evolutionary rates. *Journal of Molecular Evolution* **38**, 305–309.
- Ajzenberg, D., Bañuls, A.-L., Tibayrenc, M. and Dardé, M.L. (2002). Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *International Journal for Parasitology* **32**, 27–38.
- Alfaro, M.E., Zoller, S. and Lutzoni, F. (2003). Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**, 255–266.
- Altekar, G., Dwarkadas, S., Huelsenbeck, J.P. and Ronquist, F. (2004). Parallel Metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. *Bioinformatics* **20**, 407–415.
- Ané, C., Burleigh, J.G., McMahon, M.M. and Sanderson, M.J. (2005). Covarion structure in plastid genome evolution: a new statistical test. *Molecular Biology and Evolution* **22**, 914–924.
- Antezana, M. (2003). When being “most likely” is not enough: examining the performance of three uses of the parametric bootstrap in phylogenetics. *Journal of Molecular Evolution* **56**, 198–222.
- Arndt, P.F., Burge, C.B. and Hwa, T. (2003). DNA sequence evolution with neighbor-dependent mutation. *Journal of Computational Biology* **10**, 313–322.

- Baker, R.H., Yu, X. and DeSalle, R. (1998). Assessing the relative contribution of molecular and morphological characters in simultaneous analysis trees. *Molecular Phylogenetics and Evolution* **9**, 427–436.
- Baldauf, S.L. (2003). Phylogeny for the faint of heart: a tutorial. *Trends in Genetics* **19**, 345–351.
- Bapteste, E., Brinkmann, H., Lee, J.A., Moore, D.V., Sensen, C.W., Gordon, P., Duruflé, L., Gaasterland, T., Lopez, P., Müller, M. and Philippe, H. (2002). The analysis of 100 genes supports the grouping of three highly divergent amoebae: *Dictyostelium*, *Entamoeba*, and *Mastigamoeba*. *Proceedings of the National Academy of Sciences of the USA* **99**, 1414–1419.
- Bapteste, E. and Philippe, H. (2002). The potential value of indels as phylogenetic markers: position of trichomonads as a case study. *Molecular Biology and Evolution* **19**, 972–977.
- Barrett, M., Donoghue, M.J. and Sober, E. (1991). Against consensus. *Systematic Zoology* **40**, 486–493.
- Barriel, V. and Tassy, P. (1998). Rooting with multiple outgroups: consensus versus parsimony. *Cladistics* **14**, 193–200.
- Barta, J.R. (1997). Investigating phylogenetic relationships within the Apicomplexa using sequence data: the search for homology. *Methods* **13**, 81–88.
- Barta, J.R. (2001). Molecular approaches for inferring evolutionary relationships among protistan parasites. *Veterinary Parasitology* **101**, 175–186.
- Basáñez, M.-G., Marshall, C., Carabin, H., Gyorkos, T. and Joseph, L. (2004). Bayesian statistics for parasitologists. *Trends in Parasitology* **20**, 85–91.
- Baum, B.R. (1992). Combining trees as a way of combining data sets for phylogenetic inference, and the desirability of combining gene trees. *Taxon* **41**, 3–10.
- Baum, B.R. and Estabrook, G.F. (1996). Impact of outgroup inclusion on estimates by parsimony of undirected branching of ingroup phylogenetic lines. *Taxon* **45**, 243–257.
- Bayes, T. (1763). An essay towards solving a problem in the doctrine of chances. *Philosophical Transactions of the Royal Society of London* **53**, 370–418.
- Beaumont, M.A. and Rannala, B. (2004). The Bayesian revolution in genetics. *Nature Reviews Genetics* **5**, 251–261.
- Beebe, N.W., Cooper, R.D., Morrison, D.A. and Ellis, J.T. (2000). Subset partitioning of the ribosomal DNA small subunit and its effects on the phylogeny of the *Anopheles punctulatus* group. *Insect Molecular Biology* **9**, 515–520.
- Berry, V. and Gascuel, O. (1996). On the interpretation of bootstrap trees: appropriate threshold of clade selection and induced gain. *Molecular Biology and Evolution* **13**, 999–1011.

- Bickel, D.R. and West, B.J. (1998). Molecular evolution modeled as a fractal poisson process in agreement with mammalian sequence comparisons. *Molecular Biology and Evolution* **15**, 967–977.
- Bininda-Emonds, O.R.P. (2004). The evolution of supertrees. *Trends in Ecology and Evolution* **19**, 315–322.
- Bininda-Emonds, O.R.P., Brady, S.G., Kim, J. and Sanderson, M.J. (2001). Scaling of accuracy of extremely large phylogenetic trees. *Pacific Symposium on Biocomputing* **6**, 547–558.
- Bininda-Emonds, O.R.P., Gittleman, J.L. and Steel, M.A. (2002). The (super)tree of life: procedures, problems, and prospects. *Annual Review of Ecology and Systematics* **33**, 265–289.
- Björklund, M. (1999). Are third positions really that bad? A test using vertebrate cytochrome b. *Cladistics* **15**, 191–197.
- Blouin, C., Butt, D. and Roger, A.J. (2004). Impact of taxon sampling on the estimation of rates of evolution at sites. *Molecular Biology and Evolution* **22**, 784–791.
- Bollback, J.P. (2002). Bayesian model adequacy and choice in phylogenetics. *Molecular Biology and Evolution* **19**, 1171–1180.
- Brandley, M.C., Schmitz, A. and Reeder, T.W. (2005). Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. *Systematic Biology* **54**, 373–390.
- Brauer, M.J., Holder, M.T., Dries, L.A., Zwickl, D.J., Lewis, P.O. and Hillis, D.M. (2002). Genetic algorithms and parallel processing in maximum-likelihood phylogeny inference. *Molecular Biology and Evolution* **19**, 1717–1726.
- Bremer, B., Jansen, R.K., Oxelman, B., Backlund, M., Lantz, H. and Kim, K.-J. (1999). More characters or more taxa for a robust phylogeny—case study from the coffee family (Rubiaceae). *Systematic Biology* **48**, 413–435.
- Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**, 795–803.
- Bridge, P.D., Roberts, P.J., Spooner, B.M. and Panchal, G. (2003). On the unreliability of published DNA sequences. *New Phytologist* **160**, 43–48.
- Brower, A.V.Z., DeSalle, R. and Vogler, A. (1996). Gene trees, species trees, and systematics: a cladistic perspective. *Annual Review of Ecology and Systematics* **27**, 423–450.
- Bruno, W.J. and Halpern, A.L. (1999). Topological bias and inconsistency of maximum likelihood using wrong models. *Molecular Biology and Evolution* **16**, 564–566.
- Bryant, D. (2003). A classification of consensus methods for phylogenetics. In: *Bioconsensus* (M.F. Janowitz, F.-J. Lapointe, F.R. McMorris, B. Mirkin and F.S. Roberts, eds), pp. 163–183. Piscataway: American Mathematical Society Publications.

- Bryant, H.N. (2001). Character polarity and the rooting of cladograms. In: *The Character Concept in Evolutionary Biology* (G.P. Wagner, ed.), pp. 319–338. San Diego: Academic Press.
- Buckley, T.R., Arensburger, P., Simon, C. and Chambers, G.K. (2002). Combined data, Bayesian phylogenetics, and the origin of the New Zealand cicada genera. *Systematic Biology* **51**, 4–18.
- Buckley, T.R. and Cunningham, C.W. (2002). The effects of nucleotide substitution model assumptions on estimates of nonparametric bootstrap support. *Molecular Biology and Evolution* **19**, 394–405.
- Buckley, T.R., Simon, C. and Chambers, G.K. (2001). Exploring among-site rate variation models in a maximum likelihood framework using empirical data: effects of model assumptions on estimates of topology, branch lengths and bootstrap support. *Systematic Biology* **50**, 67–86.
- Bull, J.J., Huelsenbeck, J.P., Cunningham, C.W., Swofford, D.L. and Waddell, P.J. (1993). Partitioning and combining data in phylogenetic analysis. *Systematic Biology* **42**, 384–397.
- Butler, A.B. and Sidel, W.M. (2000). Defining sameness: historical, biological, and generative homology. *BioEssays* **22**, 846–853.
- Carpenter, J. and Bithell, J. (2000). Bootstrap confidence intervals: when, which, what? A practical guide for medical statisticians. *Statistics in Medicine* **19**, 1141–1164.
- Carreno, R.A., Martin, D.S. and Barta, J.R. (1999). *Cryptosporidium* is more closely related to the gregarines than to Coccidia as shown by phylogenetic analysis of Apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85**, 899–904.
- Castoe, T.A., Doan, T.M. and Parkinson, C.L. (2004). Data partitions and complex models in Bayesian analysis: the phylogeny of gymnophthalmid lizards. *Systematic Biology* **53**, 448–469.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**, 540–552.
- Caterino, M.S., Reed, R.D., Kuo, M.M. and Sperling, F.A.H. (2001). A partitioned likelihood analysis of swallowtail butterfly phylogeny (Lepidoptera: Papilionidae). *Systematic Biology* **50**, 106–127.
- Cerchio, S. and Tucker, P. (1998). Influence of alignment on the mtDNA phylogeny of Cetacea: questionable support for a Mysticeti Physeteroidea clade. *Systematic Biology* **47**, 336–344.
- Ceron, C., Dopazo, J., Zapata, E.L., Carazo, J.M. and Trelles, O. (1998). Parallel implementation of DNAm1 program on message-passing architectures. *Parallel Computing* **24**, 701–716.
- Chen, D., Eulenstein, O. and Fernández-Baca, D. (2004). Rainbow: a toolbox for phylogenetic supertree construction and analysis. *Bioinformatics* **20**, 2872–2873.

- Chippindale, P.T. and Wiens, J.J. (1994). Weighting, partitioning, and combining characters in phylogenetic analysis. *Systematic Biology* **43**, 278–287.
- Chor, B. and Tuller, T. (2005a). Maximum likelihood of evolutionary trees is hard. *Lecture Notes in Computer Science* **3500**, 296–310.
- Chor, B. and Tuller, T. (2005b). Maximum likelihood of evolutionary trees: hardness and approximation. *Bioinformatics* **21**(supplement), i97–i106.
- Churchill, G.A., von Haeseler, A. and Navedi, W.C. (1992). Sample size for a phylogenetic inference. *Molecular Biology and Evolution* **9**, 753–769.
- Collins, T.M., Fedrigo, O. and Naylor, G.J.P. (2005). Choosing the best genes for the job: the case for stationary genes in genome-scale phylogenetics. *Systematic Biology* **54**, 493–500.
- Conant, G.C. and Lewis, P.O. (2001). Effects of nucleotide composition bias on the success of the parsimony criterion in phylogenetic inference. *Molecular Biology and Evolution* **18**, 1024–1033.
- Cummings, M.P., Handley, S.A., Myers, D.S., Reed, D.L., Rokas, A. and Winka, K. (2003). Comparing bootstrap and posterior probability values in the four-taxon case. *Systematic Biology* **52**, 477–487.
- Cummings, M.P., Otto, S.P. and Wakeley, J. (1995). Sampling properties of DNA sequence data in phylogenetic analysis. *Molecular Biology and Evolution* **12**, 814–822.
- Cunningham, C.W. (1997a). Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**, 733–740.
- Cunningham, C.W. (1997b). Is congruence between data partitions a reliable indicator of phylogenetic accuracy? Empirically testing an iterative procedure for choosing among phylogenetic methods. *Systematic Biology* **46**, 464–478.
- Cunningham, C.W., Zhu, H. and Hillis, D.M. (1998). Best-fit maximum-likelihood models for phylogenetic inference: empirical tests with known phylogenies. *Evolution* **52**, 978–987.
- Cutler, D.J. (2000). Estimating divergence times in the presence of an over-dispersed molecular clock. *Molecular Biology and Evolution* **17**, 1647–1660.
- Davis, J.I. (1993). Character removal as a means for assessing the stability of clades. *Cladistics* **9**, 201–210.
- Davison, A.C. and Hinkley, D.V. (1997). *Bootstrap Methods and Their Applications*. Cambridge: Cambridge University Press.
- DeBry, R.W. (1999). Maximum likelihood analysis of gene-based and structure-based process partitions, using mammalian mitochondrial genomes. *Systematic Biology* **48**, 286–299.
- DeBry, R.W. (2003). Identifying conflicting signal in a multigene analysis reveals a highly resolved tree: the phylogeny of Rodentia (Mammalia). *Systematic Biology* **52**, 604–617.
- DeBry, R.W. (2005). The systematic component of phylogenetic error as a function of taxonomic sampling under parsimony. *Systematic Biology* **54**, 432–440.

- DeBry, R.W. and Olmstead, R.G. (2000). A simulation study of reduced tree-search effort in bootstrap resampling analysis. *Systematic Biology* **49**, 171–179.
- de Jong, W.W., van Dijk, M.A., Poux, C., Kappe, G., van Rheede, T. and Madsen, O. (2003). Indels in protein-coding sequences of Euarchontoglires constrain the rooting of the eutherian tree. *Molecular Phylogenetics and Evolution* **28**, 328–340.
- Delsuc, F., Brinkmann, H. and Philippe, H. (2005). Phylogenomics and the reconstruction of the tree of life. *Nature Reviews Genetics* **6**, 361–375.
- de Queiroz, A. (1993). For consensus (sometimes). *Systematic Biology* **42**, 368–372.
- de Queiroz, A., Donoghue, M.J. and Kim, J. (1995). Separate versus combined analysis of phylogenetic evidence. *Annual Review of Ecology and Systematics* **26**, 657–681.
- Dickinson, W.J. (1995). Molecules and morphology: where's the homology? *Trends in Genetics* **11**, 119–121.
- Dopazo, H., Santoyo, J. and Dopazo, J. (2004). Phylogenomics and the number of characters required for obtaining an accurate phylogeny of eukaryote model species. *Bioinformatics* **20**(supplement), i116–i121.
- Douady, C.J., Delsuc, F., Boucher, Y., Doolittle, W.F. and Douzery, E.J.P. (2003). Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. *Molecular Biology and Evolution* **20**, 248–254.
- Doyle, J.J. (1992). Gene trees and species trees: molecular systematics as one-character taxonomy. *Systematic Botany* **17**, 144–163.
- Du, Z., Stamatakis, A., Lin, F., Roshan, U. and Nakhleh, L. (2005). Parallel divide-and-conquer phylogeny reconstruction by maximum likelihood. *Lecture Notes in Computer Science* **3726**, 776–785.
- Efron, B. (1979). Bootstrapping methods: another look at the jackknife. *Annals of Statistics* **7**, 1–26.
- Efron, B., Halloran, E. and Holmes, S. (1996). Bootstrap confidence levels for phylogenetic trees. *Proceedings of the National Academy of Sciences of the USA* **93**, 7085–7090.
- Efron, B. and Tibshirani, R.J. (1993). *An Introduction to the Bootstrap*. London: Chapman & Hall.
- Erixon, P., Sennblad, B., Britton, T. and Oxelman, B. (2003). Reliability of Bayesian probabilities and bootstrap frequencies in phylogenetics. *Systematic Biology* **52**, 665–673.
- Faith, D.P. (1991). Cladistic permutation tests for monophyly and non-monophyly. *Systematic Zoology* **40**, 366–375.
- Faith, D.P. and Ballard, J.W.O. (1994). Length differences and topology-dependent tests: a response to Källersjö et al. *Cladistics* **10**, 57–64.
- Farris, J.S., Albert, V.A., Källersjö, M., Lipscomb, D. and Kluge, A.G. (1996). Parsimony jackknifing outperforms neighbor-joining. *Cladistics* **12**, 99–124.

- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* **22**, 521–565.
- Felsenstein, J. (2001). Taking variation of evolutionary rates between sites into account in inferring phylogenies. *Journal of Molecular Evolution* **53**, 447–455.
- Felsenstein, J. (2004). *Inferring Phylogenies*. Sunderland: Sinauer Associates.
- Felsenstein, J. and Churchill, G.A. (1996). A hidden Markov model approach to variation among sites in rate of evolution. *Molecular Biology and Evolution* **13**, 93–104.
- Felsenstein, J. and Kishino, H. (1993). Is there something wrong with the bootstrap on phylogenies? A reply to Hillis and Bull. *Systematic Biology* **42**, 193–200.
- Fisher, R.A. (1922). On the mathematical foundations of theoretical statistics. *Philosophical Transactions of the Royal Society of London* **222**, 309–368.
- Fitch, W.M. (2000). Homology: a personal view on some of the problems. *Trends in Genetics* **16**, 227–231.
- Fitch, W.M. and Markowitz, E. (1970). An improved method for determining codon variability in a gene and its application to the rate of fixation of mutations in evolution. *Biochemical Genetics* **4**, 579–593.
- Fleissner, R., Metzler, D. and von Haeseler, A. (2005). Simultaneous statistical multiple alignment and phylogeny reconstruction. *Systematic Biology* **54**, 548–561.
- Ford, V.S., Thomas, B.R. and Gottlieb, L.D. (1995). The same duplication accounts for the *PgiC* genes in *Clarkia xantiana* and *C. lewisii* (Onagraceae). *Systematic Botany* **20**, 147–160.
- Forster, P. (2003). To err is human. *Annals of Human Genetics* **67**, 2–4.
- Foster, P.G. (2004). Modeling compositional heterogeneity. *Systematic Biology* **53**, 485–495.
- Foster, P.G. and Hickey, D.A. (1999). Compositional bias may affect both DNA-based and protein-based phylogenetic reconstructions. *Journal of Molecular Evolution* **48**, 284–290.
- Freudenstein, J.V. (2005). Characters, states, and homology. *Systematic Biology* **54**, 965–973.
- Freudenstein, J.V. and Chase, M.W. (2001). Analysis of mitochondrial *nad1b-c* intron sequences in Orchidaceae: utility and coding of length-change characters. *Systematic Botany* **26**, 643–657.
- Gadagkar, S.R. and Kumar, S. (2005). Maximum likelihood outperforms maximum parsimony even when evolutionary rates are heterotachous. *Molecular Biology and Evolution* **22**, 2139–2141.

- Gagnon, S., Bourbeau, D. and Levesque, R.C. (1996). Secondary structures and features of the 18S, 5.8S and 26S ribosomal RNAs from the Apicomplexan parasite *Toxoplasma gondii*. *Gene* **173**, 129–135.
- Galtier, N. (2001). Maximum-likelihood phylogenetic analysis under a covarion-like model. *Molecular Biology and Evolution* **18**, 866–873.
- Galtier, N. (2004). Sampling properties of the bootstrap support in molecular phylogeny: influence of nonindependence among sites. *Systematic Biology* **53**, 38–46.
- Galtier, N. and Gouy, M. (1995). Inferring phylogenies from sequences of unequal base compositions. *Proceedings of the National Academy of Sciences of the USA* **92**, 11317–11321.
- Galtier, N. and Gouy, M. (1998). Inferring pattern and process: maximum-likelihood implementation of a nonhomogeneous model of DNA sequence evolution for phylogenetic analysis. *Molecular Biology and Evolution* **15**, 871–879.
- Gatesy, J. (2000). Linked branch support and tree stability. *Systematic Biology* **42**, 193–200.
- Gatesy, J. and Baker, R.H. (2005). Hidden likelihood support in genomic data: can forty-five wrongs make a right? *Systematic Biology* **54**, 483–492.
- Gatesy, J., Baker, R.H. and Hayashi, C. (2004). Inconsistencies in arguments for the supertree approach: supermatrices versus supertrees of Crocodylia. *Systematic Biology* **53**, 342–355.
- Gaucher, E.A., Gu, X., Miyamoto, M.M. and Benner, S.A. (2002). Predicting functional divergence in protein evolution by site-specific rate shifts. *Trends in Biochemical Sciences* **27**, 315–321.
- Germot, A. and Philippe, H. (1999). Critical analysis of eukaryotic phylogeny: a case study based on the HSP70 family. *Journal of Eukaryotic Microbiology* **46**, 116–124.
- Giribet, G. and Wheeler, W.C. (1999). On gaps. *Molecular Phylogenetics and Evolution* **13**, 132–143.
- Goldman, N. (1990). Maximum likelihood inference of phylogenetic trees, with special reference to a Poisson process model of DNA substitution and to parsimony analyses. *Systematic Zoology* **39**, 345–361.
- Goldman, N. (1993). Statistical tests of models of DNA substitution. *Journal of Molecular Evolution* **36**, 182–198.
- Goldman, N. (1998). Phylogenetic information and experimental design in molecular systematics. *Proceedings of the Royal Society of London B* **265**, 1770–1786.
- Goldman, N. and Yang, Z. (1994). A codon-based model of nucleotide substitution for protein-coding DNA sequences. *Molecular Biology and Evolution* **11**, 725–736.
- Goloboff, P.A., Farris, J.S., Källersjö, M., Oxelman, B., Ramírez, M.J. and Szumik, C.A. (2003). Improvements to resampling measures of group support. *Cladistics* **19**, 324–332.

- Gontcharov, A.A., Marin, B. and Melkonian, M. (2004). Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and *rbcL* sequence comparisons in the Zygnematophyceae (Streptophyta). *Molecular Biology and Evolution* **21**, 612–624.
- Goodman, M., Czelusniak, J., Moore, G.W., Romero-Herrera, A.E. and Matsuda, G. (1979). Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Systematic Zoology* **28**, 132–163.
- Gordon, A.D. (1986). Consensus supertrees: the synthesis of rooted trees containing overlapping sets of labeled leaves. *Journal of Classification* **3**, 31–39.
- Gough, J. (2005). Convergent evolution of domain architectures is rare. *Bioinformatics* **21**, 1464–1471.
- Graham, R.L. and Foulds, L.R. (1982). Unlikelihood that minimal phylogenies for a realistic biological study can be constructed in reasonable computational time. *Mathematical Biosciences* **60**, 133–142.
- Graham, S.W., Reeves, P.A., Burns, A.C.E. and Olmstead, R.G. (2000). Microstructural changes in noncoding chloroplast DNA: interpretation, evolution and utility of indels and inversions in basal angiosperm phylogenetic inference. *International Journal of Plant Sciences* **161**, S83–S96.
- Grant, T. and Kluge, A.G. (2003). Data exploration in phylogenetic inference: scientific, heuristic, or neither. *Cladistics* **19**, 379–418.
- Graybeal, A. (1998). Is it better to add taxa or characters to a difficult phylogenetic problem? *Systematic Biology* **47**, 9–17.
- Gu, X., Fu, Y.-X. and Li, W.-H. (1995). Maximum likelihood estimation of the heterogeneity of substitution rate among nucleotide sites. *Molecular Biology and Evolution* **12**, 546–557.
- Gu, X. and Zhang, J. (1997). A simple method for estimating the parameter of substitution rate variation among sites. *Molecular Biology and Evolution* **14**, 1106–1113.
- Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.
- Hall, B.G. (2004). *Phylogenetic Trees Made Easy: A How-To Manual*, 2nd ed. Sunderland: Sinauer Associates.
- Hall, B.G. (2005). Comparison of the accuracies of several phylogenetic methods using protein and DNA sequences. *Molecular Biology and Evolution* **22**, 792–802.
- Halpern, A.L. and Bruno, W.J. (1998). Evolutionary distances for protein-coding sequences: modeling site-specific residue frequencies. *Molecular Biology and Evolution* **15**, 910–917.
- Harris, D.J. (2003). Can you bank on GenBank? *Trends in Ecology and Evolution* **18**, 317–319.

- Hasegawa, M., Kishino, H. and Yano, T. (1985). Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160–174.
- Håstad, O. and Björklund, M. (1998). Nucleotide substitution models and estimation of phylogeny. *Molecular Biology and Evolution* **15**, 1381–1389.
- Hendy, M.D. and Holland, B.R. (2003). Upper bounds on maximum likelihood for phylogenetic trees. *Bioinformatics* **19**(supplement), ii66–ii72.
- Hendy, M.D. and Penny, D. (1993). Spectral analysis of phylogenetic data. *Journal of Classification* **10**, 5–24.
- Hennig, W. (1966). *Phylogenetic Systematics*. Urbana: University of Illinois Press. [Translated by D.D. Davis and R. Zangerl from W. Hennig (1950). *Grundzüge einer Theorie der Phylogenetischen Systematik*. Berlin: Deutscher Zentralverlag.]
- Hickson, R.E., Simon, C. and Perrey, S.W. (2000). The performance of several multiple-sequence alignment programs in relation to secondary-structure features for an rRNA sequence. *Molecular Biology and Evolution* **17**, 530–539.
- Hillis, D.M. (1996). Inferring complex phylogenies. *Nature* **383**, 130–131.
- Hillis, D.M. (1998). Taxonomic sampling, phylogenetic accuracy, and investigator bias. *Systematic Biology* **47**, 3–8.
- Hillis, D.M. and Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**, 182–192.
- Ho, S.Y.W. and Jermini, L.S. (2004). Tracing the decay of the historical signal in biological sequence data. *Systematic Biology* **53**, 623–637.
- Holder, M. and Lewis, P.O. (2003). Phylogeny estimation: traditional and Bayesian approaches. *Nature Reviews Genetics* **4**, 275–284.
- Holland, B.R., Penny, D. and Hendy, M.D. (2003). Outgroup misplacement and phylogenetic inaccuracy under a molecular clock—a simulation study. *Systematic Biology* **52**, 229–238.
- Hollich, V., Milchert, L., Arvestad, L. and Sonnhammer, E.L.L. (2005). Assessment of protein distance measures and tree-building methods for phylogenetic tree reconstruction. *Molecular Biology and Evolution* **22**, 2257–2264.
- Holmdahl, O.J.M., Morrison, D.A., Ellis, J.T. and Huong, L.T.T. (1999). Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNA sequences. *Molecular Phylogenetics and Evolution* **11**, 27–37.
- Huelsenbeck, J.P. (2002). Testing a covariotide model of DNA substitution. *Molecular Biology and Evolution* **19**, 698–707.
- Huelsenbeck, J.P., Bollback, J.P. and Levine, A.M. (2002a). Inferring the root of a phylogenetic tree. *Systematic Biology* **51**, 32–43.
- Huelsenbeck, J.P. and Bull, J.J. (1996). A likelihood ratio test to detect conflicting phylogenetic signal. *Systematic Biology* **45**, 92–98.

- Huelsenbeck, J.P., Bull, J.J. and Cunningham, C.W. (1996a). Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11**, 152–158.
- Huelsenbeck, J.P. and Crandall, K.A. (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics* **28**, 437–466.
- Huelsenbeck, J.P., Hillis, D.M. and Jones, R. (1996b). Parametric bootstrapping in molecular phylogenetics: applications and performance. In: *Molecular Zoology: Advances, Strategies, and Protocols* (J.D. Ferraris and S.R. Palumbi, eds), pp. 19–45. New York: Wiley-Liss.
- Huelsenbeck, J.P., Hillis, D.M. and Nielsen, R. (1996c). A likelihood ratio test of monophyly. *Systematic Biology* **45**, 546–558.
- Huelsenbeck, J.P., Larget, B. and Alfaro, M.E. (2004). Bayesian phylogenetic model selection using reversible jump Markov chain Monte Carlo. *Molecular Biology and Evolution* **21**, 1123–1133.
- Huelsenbeck, J.P., Larget, B., Miller, R.E. and Ronquist, F. (2002b). Potential applications and pitfalls of Bayesian inference of phylogeny. *Systematic Biology* **51**, 673–688.
- Huelsenbeck, J.P., Larget, B. and Swofford, D. (2000). A compound Poisson process for relaxing the molecular clock. *Genetics* **154**, 1879–1892.
- Huelsenbeck, J.P. and Rannala, B. (2004). Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Systematic Biology* **53**, 904–913.
- Huelsenbeck, J.P., Ronquist, F., Nielsen, R. and Bollback, J.P. (2001). Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294**, 2310–2314.
- Hughes, A.L. and Piontkivska, H. (2003). Molecular phylogenetics of Trypanosomatidae: contrasting results from 18S rRNA and protein phylogenies. *Kinetoplastid Biology and Disease* **2**, 15.
- Jensen, J.L. and Pedersen, A.-M.K. (2000). Probabilistic models of DNA sequence evolution with context dependent rates of substitution. *Advances in Applied Probability* **32**, 499–517.
- Jermiin, L.S., Ho, S.Y.W., Ababneh, F., Robinson, J. and Larkum, A.W.D. (2004). The biasing effect of compositional heterogeneity on phylogenetic estimates may be underestimated. *Systematic Biology* **53**, 638–643.
- Jin, L. and Nei, M. (1990). Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molecular Biology and Evolution* **7**, 82–102.
- Jobb, G., von Haeseler, A. and Strimmer, K. (2004). TreeFinder: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evolutionary Biology* **4**, 18.
- Johnson, J.B. and Omland, K.S. (2004). Model selection in ecology and evolution. *Trends in Ecology and Evolution* **19**, 101–108.

- Jow, H., Hudelot, C., Rattray, M. and Higgs, P.G. (2002). Bayesian phylogenetics using an RNA substitution model applied to early mammalian evolution. *Molecular Biology and Evolution* **19**, 1591–1601.
- Jukes, T.H. and Cantor, C.R. (1969). Evolution of protein molecules. In: *Mammalian Protein Metabolism* (H.N. Munro, ed.), pp. 21–132. New York: Academic Press.
- Källersjö, M., Albert, V.A. and Farris, J.S. (1999). Homoplasy increases phylogenetic structure. *Cladistics* **15**, 91–93.
- Katoh, K., Kuma, K. and Miyata, T. (2001). Genetic algorithm-based maximum-likelihood analysis for molecular phylogeny. *Journal of Molecular Evolution* **53**, 477–484.
- Kawakita, A., Sota, T., Ascher, J.S., Ito, M., Tanaka, H. and Kato, M. (2003). Evolution and phylogenetic utility of alignment gaps within intron sequences of three nuclear genes in bumble bees (*Bombus*). *Molecular Biology and Evolution* **20**, 87–92.
- Keane, T.M., Naughton, T.J., Travers, S.A.A., McInerney, J.O. and McCormack, G.P. (2005). DPRml: distributed phylogeny reconstruction by maximum likelihood. *Bioinformatics* **21**, 969–974.
- Kearney, M. (2002). Fragmentary taxa, missing data, and ambiguity: mistaken assumptions and conclusions. *Systematic Biology* **51**, 369–381.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.
- Kimura, M. (1981). Estimation of evolutionary distances between homologous nucleotide sequences. *Proceedings of the National Academy of Sciences of the USA* **78**, 454–458.
- Kjer, K.M. (2004). Aligned 18S and insect phylogeny. *Systematic Biology* **53**, 506–514.
- Knudsen, B. and Hein, J. (1999). RNA secondary structure prediction using stochastic context-free grammars and evolutionary history. *Bioinformatics* **15**, 446–454.
- Kolaczowski, B. and Thornton, J.W. (2004). Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. *Nature* **431**, 980–984.
- Kosakovsky Pond, S.L. and Muse, S.V. (2004). Column sorting: rapid calculation of the phylogenetic likelihood function. *Systematic Biology* **53**, 685–692.
- Kosakovsky Pond, S. and Muse, S.V. (2005). Site-to-site variation of synonymous substitution rates. *Molecular Biology and Evolution* **22**, 2375–2385.
- Krzywinski, J. and Besansky, N.J. (2002). Frequent intron loss in the *white* gene: a cautionary tale for phylogeneticists. *Molecular Biology and Evolution* **19**, 362–366.

- Kumar, S. and Gadagkar, S.R. (2001). Disparity index: a simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. *Genetics* **158**, 1321–1327.
- Lake, J.A. (1994). Reconstructing evolutionary trees from DNA and protein sequences: paralinear distances. *Proceedings of the National Academy of Sciences of the USA* **91**, 1455–1459.
- Lanyon, S.M. (1985). Detecting internal inconsistencies in distance data. *Systematic Zoology* **34**, 397–403.
- Lanyon, S.M. (1988). The stochastic mode of molecular evolution: what consequences for systematic investigators? *Auk* **105**, 565–573.
- Lapointe, F.-J. and Cucumel, G. (1997). The average consensus procedure: combination of weighted trees containing identical or overlapping sets of taxa. *Systematic Biology* **34**, 397–403.
- Large, B. and Simon, D.L. (1999). Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**, 750–759.
- Larson, A. (1994). The comparison of morphological and molecular data in phylogenetic systematics. In: *Molecular Ecology and Evolution: Approaches and Applications* (B. Schierwater, B. Streit, G.P. Wagner and R. DeSalle, eds), pp. 371–390. Berlin: Birkhäuser.
- Lartillot, N. and Philippe, H. (2004). A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Molecular Biology and Evolution* **21**, 1095–1109.
- Lecointre, G. and Deleporte, P. (2005). Total evidence requires exclusion of phylogenetically misleading data. *Zoologica Scripta* **34**, 101–117.
- Lecointre, G., Philippe, H., Lê, H.L.V. and Le Guyader, H. (1993). Species sampling has a major impact on phylogenetic inference. *Molecular Phylogenetics and Evolution* **2**, 205–224.
- Lecointre, G., Philippe, H., Lê, H.L.V. and Le Guyader, H. (1994). How many species are required to resolve a phylogenetic problem? The use of a new statistical method applicable to available sequences. *Molecular Phylogenetics and Evolution* **3**, 292–309.
- Lee, M.S.Y. (2000). Tree robustness and clade significance. *Systematic Biology* **49**, 829–836.
- Lee, M.S.Y. (2001). Unalignable sequences and molecular evolution. *Trends in Ecology and Evolution* **16**, 681–685.
- Lemmon, A.R. and Milinkovitch, M.C. (2002). The metapopulation genetic algorithm: an efficient solution for the problem of large phylogeny estimation. *Proceedings of the National Academy of Sciences of the USA* **99**, 10516–10521.
- Lemmon, A.R. and Moriarty, E.C. (2004). The importance of proper model assumption in Bayesian phylogenetics. *Systematic Biology* **53**, 265–277.

- Lewis, P.O. (1998a). Maximum likelihood as an alternative to parsimony for inferring phylogeny using nucleotide sequence data. In: *Molecular Systematics of Plants II: DNA Sequencing* (D.E. Soltis, P.S. Soltis and J.J. Doyle, eds), pp. 132–163. Dordrecht: Kluwer.
- Lewis, P.O. (1998b). A genetic algorithm for maximum-likelihood phylogeny inference using nucleotide sequence data. *Molecular Biology and Evolution* **15**, 277–283.
- Lewis, P.O. (2001a). A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology* **50**, 913–925.
- Lewis, P.O. (2001b). Phylogenetic systematics turns over a new leaf. *Trends in Ecology and Evolution* **16**, 30–37.
- Li, W.-H. (1997). *Molecular Evolution*. Sunderland: Sinauer Associates.
- Li, W.-H. and Zharkikh, A. (1994). What is the bootstrap technique? *Systematic Biology* **43**, 424–430.
- Lin, Y.-H., McLenachan, P.A., Gore, A.R., Phillips, M.J., Ota, R., Hendy, M.D. and Penny, D. (2002). Four new mitochondrial genomes and the increased stability of evolutionary trees of mammals from improved taxon sampling. *Molecular Biology and Evolution* **19**, 2060–2070.
- Lloyd, D.G. and Calder, V.L. (1991). Multi-residue gaps, a class of molecular characters with exceptional reliability for phylogenetic analyses. *Journal of Evolutionary Biology* **4**, 9–21.
- Lockhart, P.J., Steel, M.A., Barbrook, A.C., Huson, D.H., Charleston, M.A. and Howe, C.J. (1998). A covariotide model explains apparent phylogenetic structure of oxygenic photosynthetic lineages. *Molecular Biology and Evolution* **15**, 1183–1188.
- Lockhart, P.J., Steel, M.A., Hendy, M.D. and Penny, D. (1994). Recovering evolutionary trees under a more realistic model of sequence evolution. *Molecular Biology and Evolution* **11**, 605–612.
- Lombard, V., Camon, E.B., Parkinson, H.E., Hingamp, P., Stoesser, G. and Redaschi, N. (2002). EMBL-Align: a new public nucleotide and amino acid multiple sequence alignment database. *Bioinformatics* **18**, 763–764.
- Lopez, F.A., Manglicmot, J., Schmidt, T.M., Yeh, C., Smith, H.V. and Relman, D.A. (1999). Molecular characterization of *Cyclospora*-like organisms from baboons. *Journal of Infectious Diseases* **179**, 670–676.
- Lopez, P., Casane, D. and Philippe, H. (2002). Heterotachy, an important process of protein evolution. *Molecular Biology and Evolution* **19**, 1–7.
- Lunter, G. and Hein, J. (2004). A nucleotide substitution model with nearest-neighbour interactions. *Bioinformatics* **20**(supplement), i216–i223.
- Lunter, G., Miklós, I., Drummond, A., Jensen, J.L. and Hein, J. (2005). Bayesian coestimation of phylogeny and sequence alignment. *BMC Bioinformatics* **6**, 83.
- Lutzoni, F., Wagner, P., Reeb, V. and Zoller, S. (2000). Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. *Systematic Biology* **49**, 628–651.

- Lyons-Weiler, J. and Takahashi, K. (1999). Branch length heterogeneity leads to nonindependent branch length estimates and can decrease the efficiency of methods of phylogenetic inference. *Journal of Molecular Evolution* **49**, 392–405.
- Maddison, D.R. and Maddison, W.P. (2000). *MacClade: Analysis of Phylogeny and Character Evolution*. Sunderland: Sinauer Associates.
- Mar, J.C., Harlow, T.J. and Ragan, M.A. (2005). Bayesian and maximum likelihood phylogenetic analyses of protein sequence data under relative branch-length differences and model violation. *BMC Evolutionary Biology* **5**, 8.
- McCutchan, T.F., de la Cruz, V.F., Lal, A.A., Gunderson, J.H., Elwood, H.J. and Sogin, M.L. (1988). Primary sequences of two small subunit ribosomal RNA genes from *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **28**, 63–68.
- McGuire, G., Denham, M.C. and Balding, D.J. (2001). Models of sequence evolution for DNA sequences containing gaps. *Molecular Biology and Evolution* **18**, 281–290.
- McMorris, F.R. (1985). Axioms for consensus functions on undirected phylogenetic trees. *Mathematical Biosciences* **74**, 17–21.
- Miklós, I., Lunter, G.A. and Holmes, I. (2004). A “long indel” model for evolutionary sequence alignment. *Molecular Biology and Evolution* **21**, 529–540.
- Milinkovitch, M.C. and Lyons-Weiler, J. (1998). Finding optimal ingroup topologies and convexities when the choice of outgroups is not obvious. *Molecular Phylogenetics and Evolution* **9**, 348–357.
- Minh, B.Q., Vinh, L.S., von Haeseler, A. and Schmidt, H.A. (2005). pI-QPNNI: parallel reconstruction of large maximum likelihood phylogenies. *Bioinformatics* **21**, 3794–3796.
- Minin, V., Abdo, Z., Joyce, P. and Sullivan, J. (2003). Performance-based selection of likelihood models for phylogeny estimation. *Systematic Biology* **52**, 674–683.
- Mitchell, A., Mitter, C. and Regier, J.C. (2000). More taxa or more characters revisited: combining data from nuclear protein-encoding genes for phylogenetic analyses of Noctuoidea (Insects: Lepidoptera). *Systematic Biology* **49**, 202–224.
- Mooers, A.Ø. and Holmes, E.C. (2000). The evolution of base composition and phylogenetic inference. *Trends in Ecology and Evolution* **15**, 365–369.
- Morell, V. (1996). TreeBASE: the roots of phylogeny. *Science* **273**, 569.
- Morrison, D.A. (1996). Phylogenetic tree-building. *International Journal for Parasitology* **26**, 589–617.
- Morrison, D.A. and Ellis, J.T. (1997). Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18S rDNAs of Apicomplexa. *Molecular Biology and Evolution* **14**, 428–441.

- Morrison, D.A., Bornstein, S., Thebo, P., Wernery, U., Kinne, J. and Mattsson, J.G. (2004). The current status of the small subunit rRNA phylogeny of the Coccidia (Sporozoa). *International Journal for Parasitology* **34**, 501–514.
- Mort, M.E., Soltis, P.S., Soltis, D.E. and Mabry, M.L. (2000). Comparison of three methods for estimating internal support on phylogenetic trees. *Systematic Biology* **49**, 160–171.
- Mossel, E. and Vigoda, E. (2005). Phylogenetic MCMC algorithms are misleading on mixtures of trees. *Science* **309**, 2207–2209.
- Mu, J., Joy, D.A., Duan, J., Huang, Y., Carlton, J., Walker, J., Barnwell, J., Beerli, P., Charleston, M.A., Pybus, O.G. and Su, X.-Z. (2005). Host switch leads to emergence of *Plasmodium vivax* malaria in humans. *Molecular Biology and Evolution* **22**, 1686–1693.
- Mugridge, N.B., Morrison, D.A., Jäkel, T., Heckeroth, A.R., Tenter, A.M. and Johnson, A.M. (2000). Effects of sequence alignment and structural domains of ribosomal DNA on phylogeny reconstruction for the protozoan family Sarcocystidae. *Molecular Biology and Evolution* **17**, 1842–1853.
- Muse, S.V. (1995). Evolutionary analyses of DNA sequences subject to constraints on secondary structure. *Genetics* **139**, 1429–1439.
- Muse, S.V. and Gaut, B.S. (1994). A likelihood approach for comparing synonymous and nonsynonymous substitution rates, with application to the chloroplast genome. *Molecular Biology and Evolution* **11**, 715–724.
- Neff, N.A. (1987). An analysis of the sensitivity of minimum length (Wagner) tree topology to changes in data. *Systematic Zoology* **36**, 227–236.
- Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.
- Nei, M., Kumar, S. and Takahashi, K. (1998). The optimization principle in phylogenetic analysis tends to give incorrect topologies when the number of nucleotides or amino acids used is small. *Proceedings of the National Academy of Sciences of the USA* **95**, 12390–12397.
- Nei, M., Stevens, J.C. and Saitou, M. (1985). Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. *Molecular Biology and Evolution* **2**, 66–85.
- Newton, M.A. (1996). Bootstrapping phylogenies: large deviations and dispersion effects. *Biometrika* **83**, 315–328.
- Nielsen, R. and Yang, Z. (1998). Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. *Genetics* **148**, 929–936.
- Nixon, K.C. and Carpenter, J.M. (1993). On outgroups. *Cladistics* **9**, 413–426.

- Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P. and Nieves-Aldrey, J.L. (2004). Bayesian phylogenetic analysis of combined data. *Systematic Biology* **53**, 47–67.
- Ota, S. and Li, W.-H. (2000). NJML: a hybrid algorithm for the neighbor-joining and maximum-likelihood methods. *Molecular Biology and Evolution* **17**, 1401–1409.
- Ota, S. and Li, W.-H. (2001). NJML +: an extension of the NJML method to handle protein sequence data and computing software implementation. *Molecular Biology and Evolution* **18**, 1983–1992.
- Page, R.D.M. (2002). Modified mincut supertrees. *Lecture Notes in Computer Science* **2452**, 537–551.
- Pamilo, P. and Nei, M. (1988). Relationship between gene trees and species trees. *Molecular Biology and Evolution* **5**, 568–583.
- Pedersen, A.-M.K., Wiuf, C. and Christiansen, F.B. (1998). A codon-based model designed to describe lentiviral evolution. *Molecular Biology and Evolution* **15**, 1069–1081.
- Pedersen, J.S., Forsberg, R., Meyer, I.M. and Hein, J. (2004). An evolutionary model for protein-coding regions with conserved RNA structure. *Molecular Biology and Evolution* **21**, 1913–1922.
- Penny, D., Foulds, L.R. and Hendy, M.D. (1982). Testing the theory of evolution by comparing phylogenetic trees constructed from five different protein sequences. *Nature* **297**, 197–200.
- Penny, D., Lockhart, P.J., Steel, M.A. and Hendy, M.D. (1994). The role of models in reconstructing evolutionary trees. In: *Models in Phylogeny Reconstruction* (R.W. Scotland, D.J. Siebert and D.M. Williams, eds), pp. 211–230. Oxford: Clarendon Press.
- Penny, D., McComish, B.J., Charleston, M.A. and Hendy, M.D. (2001). Mathematical elegance with biochemical realism: the covarion model of molecular evolution. *Journal of Molecular Evolution* **53**, 711–723.
- Philippe, H., Lecomte, G., Lè, H.L.V. and Le Guyader, H. (1996). A critical study of homoplasy in molecular data with the use of a morphologically based cladogram, and its consequences for character weighting. *Molecular Biology and Evolution* **13**, 1174–1186.
- Philippe, H., Zhou, Y., Brinkmann, H., Rodrigue, N. and Delsuc, F. (2005). Heterotachy and long-branch attraction in phylogenetics. *BMC Evolutionary Biology* **5**, 50.
- Pickett, K.M. and Randle, C.P. (2005). Strange Bayes indeed: uniform topological priors imply non-uniform clade priors. *Molecular Phylogenetics and Evolution* **34**, 203–211.
- Poe, S. (1998a). The effect of taxonomic sampling on accuracy of phylogeny estimation: test case of a known phylogeny. *Molecular Biology and Evolution* **15**, 1086–1090.
- Poe, S. (1998b). Sensitivity of phylogeny estimation to taxonomic sampling. *Systematic Biology* **47**, 18–31.

- Poe, S. and Swofford, D.L. (1999). Taxon sampling revisited. *Nature* **398**, 299–300.
- Pol, D. (2004). Empirical problems of the hierarchical likelihood ratio test for model selection. *Systematic Biology* **53**, 949–962.
- Pollock, D.D. and Bruno, W.J. (2000). Assessing an unknown evolutionary process: effect of increasing site-specific knowledge through taxon addition. *Molecular Biology and Evolution* **17**, 1854–1858.
- Pollock, D.D., Zwickl, D.J., McGuire, J.A. and Hillis, D.M. (2002). Increased taxon sampling is advantageous for phylogenetic inference. *Systematic Biology* **51**, 664–671.
- Posada, D. (2001). The effect of branch length variation on the selection of models of molecular evolution. *Journal of Molecular Evolution* **52**, 434–444.
- Posada, D. and Buckley, T.R. (2004). Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* **53**, 793–808.
- Posada, D. and Crandall, K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Posada, D. and Crandall, K.A. (2001a). Selecting the best-fit model of nucleotide substitution. *Systematic Biology* **50**, 580–601.
- Posada, D. and Crandall, K.A. (2001b). Simple (wrong) models for complex trees: a case from Retroviridae. *Molecular Biology and Evolution* **18**, 271–275.
- Poux, C., van Rheede, T., Madsen, O. and de Jong, W.W. (2002). Sequence gaps join mice and men: phylogenetic evidence from deletions in two proteins. *Molecular Biology and Evolution* **19**, 2035–2037.
- Pupko, T., Huchon, D., Cao, Y., Okada, N. and Hasegawa, M. (2002). Combining multiple data sets in a likelihood analysis: which models are the best? *Molecular Biology and Evolution* **19**, 2294–2307.
- Ragan, M.A. (1992). Phylogenetic inference based on matrix representation of trees. *Molecular Phylogenetics and Evolution* **1**, 53–58.
- Rannala, B. (2002). Identifiability of parameters in MCMC Bayesian inference of phylogeny. *Systematic Biology* **51**, 754–760.
- Rannala, B., Huelsenbeck, J.P., Yang, Z. and Nielsen, R. (1998). Taxon sampling and the accuracy of large phylogenies. *Systematic Biology* **47**, 702–710.
- Rannala, B. and Yang, Z. (1996). Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *Journal of Molecular Evolution* **43**, 304–311.
- Ranwez, V. and Gascuel, O. (2002). Improvement of distance-based phylogenetic methods by a local maximum likelihood approach using triplets. *Molecular Biology and Evolution* **19**, 1952–1963.

- Redelings, B.D. and Suchard, M.A. (2005). Joint Bayesian estimation of alignment and phylogeny. *Systematic Biology* **54**, 401–418.
- Reeck, G.R., de Haën, C., Teller, D.C., Doolittle, R.F., Fitch, W.M., Dickerson, R.E., Chambon, P., McLachlan, A.D., Margoliash, E., Jukes, T.H. and Zuckerkandl, E. (1987). "Homology" in proteins and nucleic acids: a terminology muddle and a way out of it. *Cell* **50**, 667.
- Reeves, P.A., Friedman, P.H. and Richards, C.M. (2005). wolfPAC: building a high-performance distributed computing network for phylogenetic analysis using 'obsolete' computational resources. *Applied Bioinformatics* **4**, 61–64.
- Ren, F., Tanaka, H. and Yang, Z. (2005). An empirical examination of the utility of codon-substitution models in phylogeny reconstruction. *Systematic Biology* **54**, 808–818.
- Rodrigo, A.G. (1993). Calibrating the bootstrap test of monophyly. *International Journal for Parasitology* **23**, 507–514.
- Rodriguez, F., Oliver, J.L., Marin, A. and Medina, J.R. (1990). The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142**, 485–501.
- Rogers, J.S. and Swofford, D.L. (1998). A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Systematic Biology* **47**, 77–89.
- Rokas, A. and Carroll, S.B. (2005). More genes or more taxa? The relative contribution of gene number and taxon number to phylogenetic accuracy. *Molecular Biology and Evolution* **22**, 1337–1344.
- Rokas, A. and Holland, P.W. (2000). Rare genomic changes as a tool for phylogenetics. *Trends in Ecology and Evolution* **15**, 454–459.
- Rokas, A., Williams, B.L., King, N. and Carroll, S.B. (2003). Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature* **425**, 798–804.
- Ronquist, F. and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.
- Rooney, A.P. (2004). Mechanisms underlying the evolution and maintenance of functionally heterogeneous 18S rRNA genes in Apicomplexans. *Molecular Biology and Evolution* **21**, 1704–1711.
- Rosenberg, M.S. and Kumar, S. (2001). Incomplete taxon sampling is not a problem for phylogenetic inference. *Proceedings of the National Academy of Sciences of the USA* **98**, 10751–10756.
- Rosenberg, M.S. and Kumar, S. (2003). Heterogeneity of nucleotide frequencies among evolutionary lineages and phylogenetic inference. *Molecular Biology and Evolution* **20**, 610–621.
- Rosenberg, N.A. (2002). The probability of topological concordance of gene trees and species trees. *Theoretical Population Biology* **61**, 225–247.

- Rubinoff, D. and Holland, B.S. (2005). Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. *Systematic Biology* **54**, 952–961.
- Rydin, C. and Källersjö, M. (2002). Taxon sampling and seed plant phylogeny. *Cladistics* **18**, 485–513.
- Rzhetsky, A. (1995). Estimating substitution rates in ribosomal RNA genes. *Genetics* **141**, 771–783.
- Rzhetsky, A. and Nei, M. (1992). A simple method for estimating and testing minimum-evolution trees. *Molecular Biology and Evolution* **9**, 945–967.
- Rzhetsky, A. and Nei, M. (1995). Tests of applicability of several substitution models for DNA sequence data. *Molecular Biology and Evolution* **12**, 131–151.
- Rzhetsky, A. and Sitnikova, T. (1996). When is it safe to use an oversimplified substitution model in tree-making? *Molecular Biology and Evolution* **13**, 1255–1265.
- Saccone, C., Pesole, G. and Preparata, G. (1989). DNA microenvironments and the molecular clock. *Journal of Molecular Evolution* **29**, 407–411.
- Salemi, M. and Vandamme, A.-M., eds. (2003). *The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny*. Cambridge: Cambridge University Press.
- Salisbury, B.A. and Kim, J. (2001). Ancestral state estimation and taxon sampling density. *Systematic Biology* **50**, 557–564.
- Salter, L.A. and Pearl, D.K. (2001). Stochastic search strategy for estimation of maximum likelihood phylogenetic trees. *Systematic Biology* **50**, 7–17.
- Salzberg, S.L. and Yorke, J.A. (2005). Beware of mis-assembled genomes. *Bioinformatics* **21**, 4320–4321.
- Sanderson, M.J. (1989). Confidence limits on phylogenies: the bootstrap revisited. *Cladistics* **5**, 113–129.
- Sanderson, M.J. (1995). Objections to bootstrapping phylogenies: a critique. *Systematic Biology* **44**, 299–320.
- Sanderson, M.J. (1996). How many taxa must be sampled to identify the root node of a large clade? *Systematic Biology* **45**, 168–173.
- Sanderson, M.J. and Kim, J. (2000). Parametric phylogenetics? *Systematic Biology* **49**, 817–829.
- Sanderson, M.J. and Shaffer, H.B. (2002). Troubleshooting molecular phylogenetic analyses. *Annual Review of Ecology and Systematics* **33**, 49–72.
- Sanderson, M.J. and Wojciechowski, M.F. (2000). Improved bootstrap confidence limits in large-scale phylogenies, with an example from *Neoastragalus* (Leguminosae). *Systematic Biology* **49**, 671–685.
- Sanjuán, R. and Wróbel, B. (2005). Weighted least-squares likelihood ratio test for branch testing in phylogenies reconstructed from distance measures. *Systematic Biology* **54**, 218–229.

- Savill, N.J., Hoyle, D.C. and Higgs, P.G. (2001). RNA sequence evolution with secondary structure constraints: comparison of substitution rate models using maximum-likelihood methods. *Genetics* **157**, 399–411.
- Schmidt, H.A., Petzold, E., Vingron, M. and von Haeseler, A. (2003). Molecular phylogenetics: parallelized parameter estimation and quartet puzzling. *Journal of Parallel and Distributed Computing* **63**, 719–727.
- Schmidt, H.A., Strimmer, K., Vingron, M. and von Haeseler, A. (2002). Tree-Puzzle: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**, 502–504.
- Schöniger, M. and von Haeseler, A. (1994). A stochastic model for the evolution of autocorrelated DNA sequences. *Molecular Phylogenetics and Evolution* **3**, 240–247.
- Schöniger, M. and von Haeseler, A. (1999). Toward assigning helical regions in alignments of ribosomal RNA and testing the appropriateness of evolutionary models. *Journal of Molecular Evolution* **49**, 691–698.
- Semple, C., Daniel, P., Hordijk, W., Page, R.D.M. and Steel, M. (2004). Supertree algorithms for ancestral divergence dates and nested taxa. *Bioinformatics* **20**, 2355–2360.
- Seo, T.-K., Kishino, H. and Thorne, J.L. (2005). Incorporating gene-specific variation when inferring and evaluating optimal evolutionary tree topologies from multilocus sequence data. *Proceedings of the National Academy of Sciences of the USA* **102**, 4436–4441.
- Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Systematic Biology* **51**, 492–508.
- Siddall, M.E. (2001). Computer-intensive randomization in systematics. *Cladistics* **17**, S35–S52.
- Siddall, M.E. and Kluge, A.G. (1997). Probabilism and phylogenetic inference. *Cladistics* **13**, 313–336.
- Siepel, A. and Haussler, D. (2004). Phylogenetic estimation of context-dependent substitution rates by maximum likelihood. *Molecular Biology and Evolution* **21**, 468–488.
- Simmons, M.P. (2004). Independence of alignment and tree search. *Molecular Phylogenetics and Evolution* **31**, 874–879.
- Simmons, M.P. and Miya, M. (2004). Efficiently resolving the basal clades of a phylogenetic tree using Bayesian and parsimony approaches: a case study using mitogenomic data from 100 higher teleost fishes. *Molecular Phylogenetics and Evolution* **31**, 351–362.
- Simmons, M.P. and Ochoterena, H. (2000). Gaps as characters in sequence-based phylogenetic analysis. *Systematic Biology* **49**, 369–381.
- Simmons, M.P., Ochoterena, H. and Carr, T.G. (2001). Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analyses. *Systematic Biology* **50**, 454–462.
- Simmons, M.P., Pickett, K.M. and Miya, M. (2004). How meaningful are Bayesian support values? *Molecular Biology and Evolution* **21**, 188–199.

- Sitnikova, T., Rzhetsky, A. and Nei, M. (1995). Interior-branch and bootstrap tests of phylogenetic trees. *Molecular Biology and Evolution* **12**, 319–333.
- Šlapeta, J.R., Kyselová, I., Richardson, A.O., Modrý, D. and Lukeš, J. (2002). Phylogeny and sequence variability in the *Sarcocystis singaporensis* Zaman and Colley (1975) 1976 ssrDNA. *Parasitology Research* **88**, 810–815.
- Sluys, R. (1996). The notion of homology in current comparative biology. *Journal of Zoological Systematics and Evolutionary Research* **34**, 145–152.
- Smith, A.B. (1994). Rooting molecular trees: problems and strategies. *Biological Journal of the Linnean Society* **51**, 279–292.
- Sneath, P.H.A. (1986). Estimating uncertainty in evolutionary trees from Manhattan-distance triads. *Systematic Zoology* **35**, 470–488.
- Soltis, D.E., Soltis, P.S., Mort, M.E., Chase, M.W., Savolainen, V., Hoot, S.B. and Morton, C.M. (1998). Inferring complex phylogenies using parsimony: an empirical approach using three large DNA data sets for angiosperms. *Systematic Biology* **47**, 32–42.
- Soltis, P.S. and Soltis, D.E. (2003). Applying the bootstrap in phylogeny reconstruction. *Statistical Science* **18**, 256–267.
- Spencer, M., Susko, E. and Roger, A.J. (2005). Likelihood, parsimony, and heterogeneous evolution. *Molecular Biology and Evolution* **22**, 1161–1164.
- Stamatakis, A., Ludwig, T. and Meier, H. (2005). RAXML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* **21**, 456–463.
- Stamatakis, A., Ludwig, T., Meier, H. and Wolf, M.J. (2002). Accelerating parallel maximum likelihood-based phylogenetic tree calculations using subtree equality vectors. In: *Proceedings of the 15th IEEE/ACM Conference on Supercomputing*, pp. 1–16. New York: ACM Press.
- Steel, M. (2005). Should phylogenetic models be trying to ‘fit an elephant’? *Trends in Genetics* **21**, 307–309.
- Steel, M., Dress, A.W.M. and Böcker, S. (2000a). Simple but fundamental limitations on supertree and consensus tree methods. *Systematic Biology* **49**, 363–368.
- Steel, M., Huson, D. and Lockhart, P.J. (2000b). Invariable sites models and their use in phylogeny reconstruction. *Systematic Biology* **49**, 225–232.
- Steel, M. and Penny, D. (2000). Parsimony, likelihood, and the role of models in molecular phylogenetics. *Molecular Biology and Evolution* **17**, 839–850.
- Steinbachs, J.E., Schizas, N.V. and Ballard, J.W.O. (2001). Efficiencies of genes and accuracy of tree-building methods in recovering a known *Drosophila* genealogy. *Pacific Symposium on Biocomputing* **6**, 606–617.
- Stevens, J.R. and Schofield, C.J. (2003). Phylogenetics and sequence analysis—some problems for the unwary. *Trends in Parasitology* **19**, 582–588.

- Stewart, C.A., Hart, D., Berry, D.K., Olsen, G.J., Wernert, E.A. and Fischer, W. (2001). Parallel implementation and performance of fastDNAm1 – a program for maximum likelihood phylogenetic inference. In: *Proceedings of the 14th IEEE/ACM Conference on Supercomputing*, pp. 20–20 [sic]. New York: ACM Press.
- Stone, J. (1998). Using a nonrecursive formula to determine cladogram probabilities. *Systematic Biology* **47**, 617–624.
- Strimmer, K. and von Haeseler, A. (1996). Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution* **13**, 964–969.
- Suchard, M.A., Weiss, R.E. and Sinsheimer, J.A. (2001). Bayesian selection of continuous-time Markov chain evolutionary models. *Molecular Biology and Evolution* **18**, 1001–1013.
- Sullivan, J., Abdo, Z., Joyce, P. and Swofford, D.L. (2005). Evaluating the performance of a successive-approximations approach to parameter optimization in maximum-likelihood phylogeny estimation. *Molecular Biology and Evolution* **22**, 1386–1392.
- Sullivan, J. and Swofford, D.L. (2001). Should we use model-based methods of phylogenetic inference when we know that assumptions about among-site rate variation and nucleotide substitution pattern are violated? *Systematic Biology* **50**, 723–729.
- Sullivan, J., Swofford, D.L. and Naylor, G.J.P. (1999). The effect of taxon sampling on estimating rate heterogeneity parameters of maximum-likelihood models. *Molecular Biology and Evolution* **16**, 1347–1356.
- Sültmann, H., Mayer, W.E., Figueroa, F., Tichy, H. and Klein, J. (1995). Phylogenetic analysis of cichlid fishes using nuclear DNA markers. *Molecular Biology and Evolution* **12**, 1033–1047.
- Sumrall, C.D., Brochu, C.A. and Merck, J.W. (2001). Global lability, regional resolution, and majority-rule consensus bias. *Palaeobiology* **27**, 254–261.
- Suzuki, Y., Glazko, G.V. and Nei, M. (2002). Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. *Proceedings of the National Academy of Sciences of the USA* **99**, 16138–16143.
- Swofford, D.L. (1991). When are phylogeny estimates from molecular and morphological data incongruent? In: *Phylogenetic Analysis of DNA Sequences* (M.M. Miyamoto and J. Cracraft, eds), pp. 295–333. New York: Oxford University Press.
- Swofford, D.L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sunderland: Sinauer Associates.
- Swofford, D.L., Olsen, G.J., Waddell, P.J. and Hillis, D.M. (1996). Phylogenetic inference. In: *Molecular Systematics* (D.M. Hillis, C. Moritz and B.K. Mable, eds), 2nd ed., pp. 407–514. Sinauer Associates: Sunderland.

- Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526.
- Tarrio, R., Rodríguez-Trelles, F. and Ayala, F.J. (2000). Tree rooting with outgroups when they differ in their nucleotide composition from the ingroup: the *Drosophila saltans* and *willistoni* groups, a case study. *Molecular Phylogenetics and Evolution* **16**, 344–349.
- Tavaré, S. (1986). Some probabilistic and statistical problems on the analysis of DNA sequences. *Lectures on Mathematics in the Life Sciences* **17**, 57–86.
- Taylor, D.J. and Piel, W.H. (2004). An assessment of accuracy, error, and conflict with support values from genome-scale phylogenetic data. *Molecular Biology and Evolution* **21**, 1534–1537.
- Telford, M.J., Wise, M.J. and Gowri-Shankar, V. (2005). Consideration of RNA secondary structure significantly improves likelihood-based estimates of phylogeny: examples from Bilateria. *Molecular Biology and Evolution* **22**, 1129–1136.
- Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.
- Tillier, E.R.M. (1994). Maximum likelihood with multi-parameter models of substitution. *Journal of Molecular Evolution* **39**, 409–417.
- Tillier, E.R.M. and Collins, R.A. (1995). Neighbor joining and maximum likelihood with RNA sequences: addressing the inter-dependence of sites. *Molecular Biology and Evolution* **12**, 7–15.
- Tillier, E.R.M. and Collins, R.A. (1998). High apparent rate of simultaneous compensatory base-pair substitutions in ribosomal RNA. *Genetics* **148**, 1993–2002.
- Tuffley, C. and Steel, M. (1998a). Links between maximum likelihood and maximum parsimony under a simple model of site substitution. *Bulletin of Mathematical Biology* **59**, 581–607.
- Tuffley, C. and Steel, M. (1998b). Modeling the covarion hypothesis of nucleotide substitution. *Mathematical Biosciences* **147**, 63–91.
- van Dijk, M.A.M., Paradis, E., Catzeflis, F. and de Jong, W.W. (1999). The virtues of gaps: xenarthran (edentate) monophyly supported by a unique deletion in the alpha-A-crystalline. *Systematic Biology* **48**, 94–106.
- Vinh, L.S. and von Haeseler, A. (2004). IQPNNI: moving fast through tree space and stopping in time. *Molecular Biology and Evolution* **21**, 1565–1571.
- von Haeseler, A. and Schöniger, M. (1998). Evolution of DNA of amino acid sequences with dependent sites. *Journal of Computational Biology* **5**, 149–163.

- Vos, R.A. (2003). Accelerated likelihood surface exploration: the likelihood ratchet. *Systematic Biology* **52**, 368–373.
- Waddell, P.J., Kishino, H. and Ota, R. (2000). Rapid evaluation of the phylogenetic congruence of sequence data using likelihood ratio tests. *Molecular Biology and Evolution* **17**, 1988–1992.
- Waddell, P.J., Kishino, H. and Ota, R. (2002). Very fast algorithms for evaluating the stability of ML and Bayesian phylogenetic trees from sequence data. *Genome Informatics* **13**, 82–92.
- Waddell, P.J. and Steel, M.A. (1997). General time-reversible distances with unequal rates across sites: mixing Γ and inverse Gaussian distributions with invariant sites. *Molecular Phylogenetics and Evolution* **8**, 398–414.
- Weisburg, W.G., Giovannoni, S.J. and Woese, C.R. (1989). The *Deinococcus* and *Thermus* phylum and the effect of ribosomal RNA composition on phylogenetic tree construction. *Systematic and Applied Microbiology* **11**, 128–134.
- Wenzel, J.W. and Siddall, M.E. (1999). Noise. *Cladistics* **15**, 51–64.
- Wesseling, J.G., Snijders, P.J., van Someren, P., Jansen, J., Smits, M.A. and Schoenmakers, J.G. (1989). Stage-specific expression and genomic organization of the actin genes of the malaria parasite *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **35**, 167–176.
- Wheeler, W.C. (1990). Nucleic acid sequence phylogeny and random outgroups. *Cladistics* **6**, 363–367.
- Wheeler, W. (1996). Optimization alignment: the end of multiple sequence alignment in phylogenetics? *Cladistics* **12**, 1–9.
- Wheeler, W.C. (1999). Fixed character states and the optimization of molecular sequence data. *Cladistics* **15**, 379–385.
- Wheeler, W.C., Gatesy, J. and DeSalle, R. (1995). Elision: a method for accommodating multiple molecular sequence alignments with alignment-ambiguous sites. *Molecular Phylogenetics and Evolution* **4**, 1–9.
- Whelan, S., Liò, P. and Goldman, N. (2001). Molecular phylogenetics: state-of-the-art methods for looking into the past. *Trends in Genetics* **17**, 262–272.
- Wiens, J.J. (1998). Does adding characters with missing data increase or decrease phylogenetic accuracy? *Systematic Biology* **47**, 625–640.
- Wiens, J.J. (2005). Can incomplete taxa rescue phylogenetic analyses from long-branch attraction? *Systematic Biology* **54**, 731–742.
- Wiens, J.J., Fetzner, J.W., Parkinson, C.L. and Reeder, T.W. (2005). Hylid frog phylogeny and sampling strategies for speciose clades. *Systematic Biology* **54**, 778–807.
- Wilcox, T.P., Zwickl, D., Heath, T.A. and Hillis, D.M. (2002). Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Molecular Phylogenetics and Evolution* **25**, 361–371.

- Wilkinson, M. (1994). Common cladistic information and its consensus representation: reduced Adams and reduced cladistic consensus trees and profiles. *Systematic Biology* **43**, 343–368.
- Wilkinson, M. (1995a). More on reduced consensus methods. *Systematic Biology* **44**, 435–439.
- Wilkinson, M. (1995b). Coping with abundant missing entries in phylogenetic inference using parsimony. *Systematic Biology* **44**, 501–514.
- Wilkinson, M., Cotton, J.A., Creevey, C., Eulenstein, O., Harris, S.R., Lapointe, F.-J., Levasseur, C., McInerney, J.O., Pisani, D. and Thorley, J.L. (2005). The shape of supertrees to come: tree shape related properties of fourteen supertree methods. *Systematic Biology* **54**, 419–431.
- Williams, T.L. and Moret, B.M.E., 2003. An investigation of phylogenetic likelihood methods. In: *Proceedings of the 3rd IEEE Symposium on Bioinformatics and Bioengineering*, pp. 79–86. Piscataway: IEEE Press.
- Woodhams, M.D. and Hendy, M.D. (2004). Reconstructing phylogeny by quadratically approximated maximum likelihood. *Bioinformatics* **20**(supplement), i348–i354.
- Wortley, A.H., Rudall, P.A., Harris, D.J. and Scotland, R.W. (2005). How much data are needed to resolve a difficult phylogeny? Case study in Lamiales. *Systematic Biology* **54**, 697–709.
- Wuyts, J., Perrière, G. and Van de Peer, Y. (2004). The European ribosomal RNA database. *Nucleic Acids Research* **32**, D101–D103.
- Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R. and Lal, A.A. (2002). Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. *International Journal for Parasitology* **32**, 1773–1785.
- Yang, Z. (1993). Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution* **10**, 1396–1401.
- Yang, Z. (1994). Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *Journal of Molecular Evolution* **39**, 306–314.
- Yang, Z. (1995). A space-time process model for the evolution of DNA sequences. *Genetics* **139**, 993–1005.
- Yang, Z. (1996a). Among-site rate variation and its impact on phylogenetic analyses. *Trends in Ecology and Evolution* **11**, 367–372.
- Yang, Z. (1996b). Maximum-likelihood models for combined analyses of multiple sequence data. *Journal of Molecular Evolution* **42**, 587–596.
- Yang, Z. (1997a). How often do wrong models produce better phylogenies? *Molecular Biology and Evolution* **14**, 105–108.
- Yang, Z. (1997b). PAML: a program package for phylogenetic analysis by maximum likelihood. *Computer Applications in the Biosciences* **13**, 555–556.

- Yang, Z. (1998). On the best evolutionary rate for phylogenetic analysis. *Systematic Biology* **47**, 125–133.
- Yang, Z. (2000). Complexity of the simplest phylogenetic estimation problem. *Proceedings of the Royal Society of London B* **267**, 109–116.
- Yang, Z. and Goldman, N. (1997). Are big trees indeed easy? *Trends in Ecology and Evolution* **12**, 357.
- Yang, Z., Goldman, N. and Friday, A.E. (1995). Maximum likelihood trees from DNA sequences: a peculiar statistical estimation problem. *Systematic Biology* **44**, 384–399.
- Yang, Z. and Nielsen, R. (2002). Codon-substitution models for detecting molecular adaptation at individual sites along individual lineages. *Molecular Biology and Evolution* **19**, 908–917.
- Yang, Z., Nielsen, R., Goldman, N. and Pedersen, A.-M.K. (2000). Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* **155**, 431–449.
- Yang, Z. and Rannala, B. (2005). Branch-length prior influences Bayesian posterior probability of phylogeny. *Systematic Biology* **54**, 455–470.
- Yang, Z. and Roberts, D. (1995). On the use of nucleic acid sequences to infer early branchings in the tree of life. *Molecular Biology and Evolution* **12**, 451–458.
- Yang, Z. and Swanson, W.J. (2002). Codon-substitution models to detect adaptive evolution that account for heterogeneous selective pressures among site classes. *Molecular Biology and Evolution* **19**, 49–57.
- Yang, Z. and Yoder, A.D. (1999). Estimation of the transition/transversion rate bias and species sampling. *Journal of Molecular Evolution* **48**, 274–283.
- Yap, V.B. and Speed, T. (2005). Rooting a phylogenetic tree with nonreversible substitution models. *BMC Evolutionary Biology* **5**, 2.
- Young, N.D. and Healy, J. (2003). GapCoder automates the use of indel characters in phylogenetic analysis. *BMC Bioinformatics* **4**, 6.
- Zander, R.H. (2004). Minimal values for reliability of bootstrap and jack-knife proportions, decay index, and Bayesian posterior probability. *PhyloInformatics* **1**(2), 1–13.
- Zharkikh, A. (1994). Estimation of evolutionary distances between nucleotide sequences. *Journal of Molecular Evolution* **39**, 315–329.
- Zharkikh, A. and Li, W.-H. (1992a). Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. I. Four taxa with a molecular clock. *Molecular Biology and Evolution* **9**, 1119–1147.
- Zharkikh, A. and Li, W.-H. (1992b). Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. II. Four taxa without a molecular clock. *Journal of Molecular Evolution* **35**, 356–366.

- Zharkikh, A. and Li, W.-H. (1995). Estimation of confidence in phylogeny: the complete-and-partial bootstrap technique. *Molecular Phylogenetics and Evolution* **4**, 44–63.
- Zhu, G., Keithly, J.S. and Philippe, H. (2000). What is the phylogenetic position of *Cryptosporidium*? *International Journal of Systematic and Evolutionary Microbiology* **50**, 1673–1681.
- Zwickl, D.J. and Hillis, D.M. (2002). Increased taxon sampling greatly reduces phylogenetic error. *Systematic Biology* **51**, 588–598.
- Zwickl, D.J. and Holder, M.T. (2004). Model parameterization, prior distributions, and the general time-reversible model in Bayesian phylogenetics. *Systematic Biology* **53**, 877–888.

Targeting of Toxic Compounds to the Trypanosome's Interior

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ABSTRACT

Drugs can be targeted into African trypanosomes by exploiting carrier proteins at the surface of these parasites. This has been clearly demonstrated in the case of the melamine-based arsenical and the diamidine classes of drug that are already in use in the treatment of human African trypanosomiasis. These drugs can enter via an aminopurine transporter, termed P2, encoded by the *ThAT1* gene. Other toxic compounds have also been designed to enter via this transporter. Some of these compounds enter almost exclusively through the P2 transporter, and hence loss of the P2 transporter leads to significant resistance to these particular compounds. It now appears, however, that some diamidines and melaminophenylarsenicals may also be taken up by other routes (of yet unknown function). These too may be exploited to target new drugs into trypanosomes. Additional purine nucleoside and nucleobase transporters have also been subverted to deliver toxic agents to trypanosomes. Glucose and amino acid transporters too have been investigated with a view to manipulating them to carry toxins into *Trypanosoma brucei*, and recent work has demonstrated that aquaglyceroporins may also have considerable potential for drug-targeting. Transporters, including those that carry lipids and vitamins such as folate and other pterins also deserve more attention in this regard. Some drugs, for example suramin, appear to enter via routes other than plasma-membrane-mediated transport. Receptor-mediated endocytosis has been proposed as a possible way in for suramin. Endocytosis also appears to be crucial in targeting natural trypanocides, such as trypanosome lytic factor (TLF) (apolipoprotein L1), into trypanosomes and this offers an alternative means of selectively targeting toxins to the trypanosome's interior. Other compounds may be induced to enter by increasing their capacity to diffuse over cell membranes; in this case depending exclusively on selective activity within the cell rather than selective uptake to impart selective toxicity. This review outlines studies that have aimed to exploit trypanosome nutrient uptake routes to selectively carry toxins into these parasites.

1. INTRODUCTION

Human African trypanosomiasis (HAT), caused by African trypanosomes of the brucei group, has become resurgent in Africa (Barrett *et al.*, 2003). Two sub-species of *Trypanosoma brucei* are recognised as causing human disease. In Eastern and Southern Africa *T. b. rhodesiense* causes an acute form of the disease while in West and Central Africa *T. b. gambiense* causes a chronic form. While the rhodesiense form parasites caused some major epidemics at the beginning of the twentieth century (Fevre *et al.*, 2004), it is the gambiense form that was responsible for the epidemics that marked the end of that century. It is critical that measures to curb the problem are in place. Vector control has the potential to offer a long-term solution; however, this is only relevant if it can be sustained (Allsopp, 2001) and for the 300 000 or so individuals currently infected this is of little use. No vaccines exist against HAT, the prospects of prophylactic immunisation being poor because of the well-documented process of antigenic variation (McCulloch, 2004). Current drugs (Pépin and Milord, 1994; Barrett, 2000; Legros *et al.*, 2002; Fairlamb, 2003), the principal means of intervention, also suffer drawbacks due to toxicity, resistance and lack of a guaranteed supply. The only compound in advanced trials is the orally available prodrug, DB289 (Ansede *et al.*, 2004), which is converted systemically to the diamidine DB75, a compound active only against early stage disease. Other agents are urgently required to improve the arsenal of available trypanocides.

Selective toxicity against any microorganism can be achieved through various means. These include selective binding of drug to a specific microbial target, through a common target being vital to a microbe but not a host cell, or through the selective uptake of drugs into pathogens. Since trypanosomes are extracellular parasites, any toxic compound which is selectively permeable to the parasite membrane will exert an activity against the parasite regardless of whether its intracellular targets are shared between parasite and host. Paul Ehrlich, "the father of chemotherapy" whose pioneering work demonstrated the potential of synthetic chemicals to kill microbes (Drews, 2004), himself reckoned that most drugs should comprise toxophilic and haptophoric moieties; the toxic moiety being carried into cells via

the haptophoric moiety. It was upon this guiding principle that Nicola Carter and Alan Fairlamb first gained insight into the utility of the P2 aminopurine transporter to carry drugs into trypanosomes (Carter and Fairlamb, 1993) in what might be considered a watershed in re-establishing the notion that selective toxicity in trypanosomes might be induced by selective uptake of toxins.

As parasites, which by definition “feed at the table of another” (“parasitos” taken from the Greek translates roughly to this), trypanosomes depend on their hosts for a number of vital nutrients. In the case of bloodstream form trypanosomes that live free in the bloodstream or other fluids of their hosts, including the cerebrospinal fluid, the uptake of a number of nutrients including glucose, purines, some amino acids and vitamins and lipids is essential (Hasne and Barrett, 2000a; de Koning, 2001a; Maser *et al.*, 2003; Zilberstein, 1993). In order to scavenge these molecules, the parasites are replete with plasma membrane transporters and channels and also with receptors that mediate endocytosis of selected nutrients. A number of these have been studied at the biochemical and molecular levels. The recent publication of the *T. brucei* genome sequence (Berriman *et al.*, 2005) has highlighted many more whose function is yet to be established.

All of these pathways of nutrient uptake can potentially be subverted to carry toxins selectively into trypanosomes, leading to the possibility of selective parasite toxicity through selective uptake. Some transporters will turn out to be more useful for this strategy than others. This review outlines those strategies that have been employed towards this goal.

2. UPTAKE AND ACTION OF CURRENT TRYPANOCIDAL DRUGS

Before considering how new compounds may be targeted into trypanosomes we will first review what is known about uptake and selectivity of those drugs already registered or under consideration for use against HAT (Figure 1). Four licensed compounds are used, depending on the causative subspecies and whether parasites have initiated disease of the central nervous system (late stage) or not

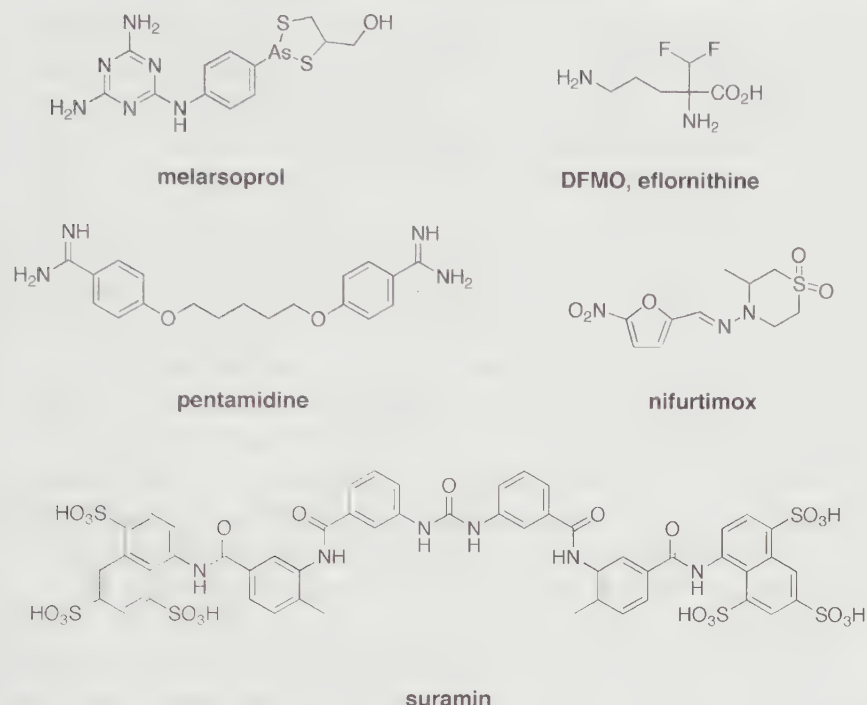


Figure 1 Structures of drugs currently used in HAT therapy. Chemical structures for those drugs currently licensed for use against human African trypanosomiasis, along with nifurtimox which is in trials as an agent to use in combination with other trypanocides and is licensed for treating American trypanosomiasis.

(early stage) (Pépin and Milord, 1994; Keiser *et al.*, 2001; Denise and Barrett, 2001; Fairlamb, 2003). Two compounds are used against early stage disease, suramin and pentamidine. Against late-stage disease, melarsoprol (active against *T. b. gambiense* and *T. b. rhodesiense*) and eflornithine (only useful against *T. b. gambiense*) can be used. Nifurtimox, alone or in combination with other drugs, is being considered as an option for melarsoprol-refractory late-stage disease.

2.1. Melarsoprol

Melarsoprol is a melaminophenyl-based organic arsenical, which was introduced as an anti-trypanosomiasis reagent in 1949. Of all the

current drugs registered for HAT, toxic effects are worst with melarsoprol; up to 10% of patients suffer a frequently fatal reactive encephalopathy. Melarsoprol itself is amphipathic and will diffuse across cellular membranes (lipophilic compounds like melarsoprol will preferentially partition into the hydrophobic environment of the lipid bilayer; the local concentration in this compartment will rise and at a level dependent upon the partition coefficient of the given compound it will be released into neighbouring compartments). However, melarsoprol is very rapidly converted to the relatively hydrophilic melarsen oxide in plasma (96% clearance of melarsoprol within 1 h) (Burri *et al.*, 1993). Melarsen oxide levels peak within 15 min and have a half-life of 3.9 hours. Melarsoprol or its metabolites maximally accumulate across the blood-brain barrier to levels only around 1–2% of maximum plasma levels. This is sufficient to kill wild-type trypanosomes, but it should be considered that levels of drug that reach the cerebrospinal fluid might be insufficient to kill parasites that are only a few fold less sensitive to drugs than wild-type (de Koning, 2001a).

In vitro, unmetabolised melarsoprol is likely to cross membranes by passive diffusion (Scott *et al.*, 1997) although melarsen oxide does not. Melarsen oxide has been proposed to enter *T. brucei* by the P2 amino-purine transporter (Carter and Fairlamb, 1993; Maser *et al.*, 1999) although other transporters too are probably capable of carrying the drug (Matovu *et al.*, 2003).

A definite mode of action for melarsoprol is yet to be established. Trypanosomes exposed to arsenicals lyse rapidly. Loss of ATP due to inhibition of glycolysis could underlie lysis as the bloodstream form trypanosomes depend solely upon glycolysis for ATP production. However, it seems that the cells lyse before ATP supplies are seriously depleted leading several workers to question whether glycolysis is the target for arsenical action (Vanschafingen *et al.*, 1987). Since arsenic is known to form stable interactions with thiols, another suggested target of melarsoprol is trypanothione (Fairlamb *et al.*, 1989), a key low molecular weight thiol found in trypanosomatids but not in mammalian cells (Fairlamb *et al.*, 1985). Trypanothione makes tight conjugates with melarsoprol (Fairlamb *et al.*, 1989). Interestingly, other thiols such as lipoic acid form even tighter conjugates with the drug (Fairlamb *et al.*, 1992b).

Treatment failure with melarsoprol has been reported in the field. There has always been a cohort of 5–10% of treated patients who relapsed after treatment, although it was never clear what factors were responsible for this. In several foci, this has reached levels of 30% (Legros *et al.*, 1999; Brun *et al.*, 2001; Moore and Richer, 2001; Stanghellini and Josenando, 2001) although the reasons for this are not yet clear. A number of parasites selected for resistance to melamine-based arsenicals in the laboratory have lost the P2 transporter (Carter and Fairlamb, 1993; Maser *et al.*, 1999; Stewart *et al.*, 2005), indicating that loss of this transporter can contribute to development of resistance. Several parasites isolated from relapse cases in the field are also defective in P2 transport (Maser *et al.*, 1999; Matovu *et al.*, 2001; Stewart *et al.*, 2005) and it has been possible to develop a fluorescence-based test for loss of P2 that may represent a good diagnostic test for resistance in the field (Stewart *et al.*, 2005). The test depends on the fact that some fluorescent diamidines enter trypanosomes principally through the P2 transporter that also carries melarsoprol and whose activity is lost in melarsoprol resistance. Thus loss of transporter correlates with delayed development of fluorescence in these parasites.

In spite of the clear correlation of loss of P2 to resistance, genetically modified trypanosomes that were deleted in the *TbAT1* gene that encodes the transporter (Maser *et al.*, 1999) showed only around two- to threefold reduced sensitivity to melarsen oxide (Matovu *et al.*, 2003). The drug must therefore also enter via other routes and additional mechanisms must be at play in determining high-level resistance in laboratory-derived lines. The rapid lysis of trypanosomes by arsenicals can be followed spectrophotometrically (Meshnick *et al.*, 1978) as living cells scatter light at 600 nm whilst this scatter is lost when cells lyse. It was using this assay that Carter and Fairlamb were first able to show that the P2 aminopurine transporter was involved in uptake of the melaminophenylarsenicals (Carter and Fairlamb, 1993). The same assay was able to show a secondary, adenosine-independent, transporter that could also allow delayed lysis in cells lacking the P2 transporter (Matovu *et al.*, 2003). Since this transporter was inhibited by low concentrations of pentamidine it was proposed (Matovu *et al.*, 2003) that melarsoprol might also enter via the high-affinity pentamidine transporter (HAPT1) (de Koning and

Jarvis, 2001; de Koning, 2001b). A modest change in sensitivity relating to impairment of P2 function, however, could still render parasites resistant to levels of melarsen oxide accumulating in the CSF and other extravascular compartments where the drug reaches relatively low levels (Burri *et al.*, 1993). In summary, current data indicate that, *in vivo*, melarsoprol is rapidly metabolised to melarsen oxide. Uptake into trypanosomes of this metabolite occurs principally through the P2 aminopurine transporter and probably at least one additional carrier-mediated route.

2.2. Pentamidine

Pentamidine is an aromatic diamidine that has been in use for treatment of trypanosomiasis for over 50 years (Sands *et al.*, 1985). Maximum plasma concentrations are reached within an hour of intramuscular injection. Variation is found in plasma concentrations between individuals ($0.2\text{--}4.4\text{ mg L}^{-1}$ following a 4 mg kg^{-1} injection) and each daily dose leads to an increase in residual drug concentration. Elimination is slow with estimates of 70–80% of the drug reported to bind to plasma proteins. The average plasma half-life is 12 days (but varies) and the drug is extensively metabolised by humans (only around 11% is eliminated in the urine). The drug, at most, only crosses the blood brain barrier to a very small extent, although there are reports suggesting small amounts may enter the cerebrospinal fluid and the drug might be curative in the “early late-stage disease” when parasites have only recently appeared in the CSF (Doua *et al.*, 1996).

Pentamidine is concentrated to high levels by trypanosomes (Carter *et al.*, 1995; Damper and Patton, 1976a, b; Berger *et al.*, 1995; de Koning, 2001a; Bray *et al.*, 2003). The drug enters *T. brucei* via the same P2 aminopurine transporter, which accumulates melaminophenyl arsenicals (Carter *et al.*, 1995). This is because the P2 recognition motif is also found in diamidines (Figure 2). However, while arsenical pentamidine cross resistance has been noted (Frommel and Balber, 1987) parasites deficient in the P2 transporter generally remain sensitive to pentamidine (Fairlamb *et al.*, 1992a; Matovu

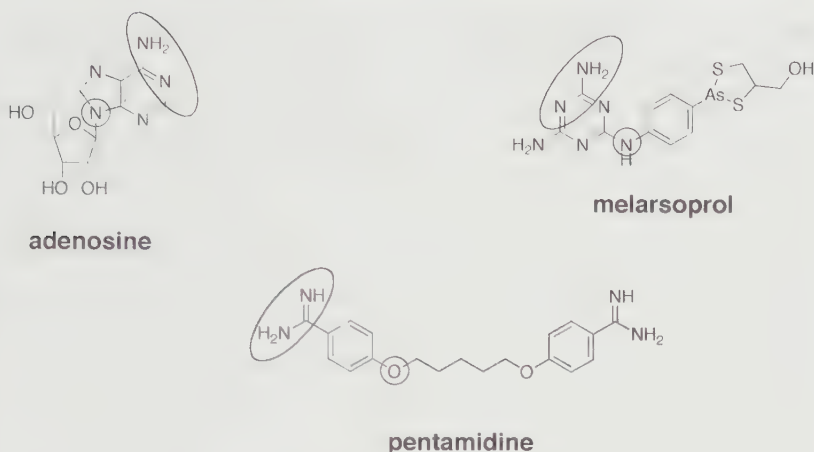


Figure 2 The P2 recognition motif. The motif recognised by the P2 transporter in adenosine, pentamidine and melarsoprol is highlighted with a ring around the primary motif. An aromatic ring structure and electro-negative heteroatom (also ringed) appear to be necessary too.

et al., 2003; de Koning, 2001b). It has been shown that this is probably because in *T. brucei* at least three transporters that can carry pentamidine into the cell have been identified (de Koning, 2001b). In addition to P2, a high-affinity transporter HAPT1 (K_m for pentamidine = 36 nM) and a low-affinity transporter LAPT1 (K_m for pentamidine = 56 μ M) also contribute to the uptake of pentamidine. The mode of action of the drug has not been established (Berger *et al.*, 1993). As a polycation, pentamidine interacts electrostatically with cellular polyanions. It binds DNA, including the unique intercatenated network of circular DNA molecules which make up the mitochondrial genome of all kinetoplastid flagellates termed the kinetoplast (Simpson, 1986). *T. brucei* can retain viability, given time to adapt, when the kinetoplast has disintegrated (a state termed dyskinetoplasty; Schnauffer *et al.*, 2002) although mitochondrial DNA can remain dispersed within the mitochondrion in some of these cases. On the other hand, dyskinetoplastic parasites have been shown to be somewhat less sensitive than wild-type cells to another diamidine, diminazene (Agbe and Yielding, 1995). Fluorescent analogues of the diamidines, e.g. DB75 (Stewart *et al.*, 2005), and stilbamidine (Hawking and Smiles, 1941) accumulate rapidly in the

kinetoplast, and have also been shown to accumulate in small vesicular structures in the cytosol.

Pentamidine resistance did not emerge on a significant scale during large-scale chemoprophylaxis campaigns in the middle part of the twentieth century in West Africa (Bray *et al.*, 2003), indicating that it may be difficult to select stable resistance to this drug. It is probably due to the fact that pentamidine can continue to enter via other transporters once P2 is lost (de Koning, 2001b) that has made the selection of resistance to this drug so difficult. One laboratory-induced line with resistance to pentamidine was shown to continue to accumulate drug to high levels and retain activity of the P2 transporter (Berger *et al.*, 1995). Interestingly, this parasite line was of much reduced virulence in rodents, which would indicate that the development of resistance to pentamidine might be associated with very substantial fitness costs, rendering the propagation of resistant lines in the field unlikely (Berger *et al.*, 1995; Bray *et al.*, 2003).

2.3. Diminazene

Diminazene is widely used as a veterinary trypanocide (Kinabo, 1993; Peregrine and Mamman, 1993). Its use in humans has been attempted; however, concerns about safety and also the prospect of selecting resistance that contributes to cross resistance involving other drugs have been raised (Barrett, 2001; Bray *et al.*, 2003). Diminazene is a diamidine (Figure 3) and the drug was first reported to enter via the P2 transporter when it was shown that lines of *T. equiperdum* (see Barrett *et al.*, 1995b) and *T. evansi* (see Ross and Barns, 1996) lines selected for diminazene resistance and cymelarsan resistance, respectively, had lost that transporter. Uptake via the P2 transporter was confirmed using ^3H -diminazene and it was revealed that the P2 transporter was the principal route for this drug (de Koning *et al.*, 2004). TbAT1 knockout cells (Matovu *et al.*, 2003) are highly resistant to diminazene in short term *in vitro* assays again indicating that the P2 transporter is the principal route of uptake of this drug. RNA interference was used to knockdown expression in *T. evansi* of TeAT1, which also yielded high-level resistance to diminazene (Witola

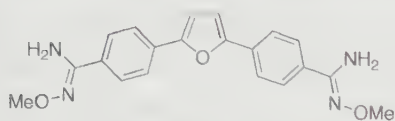
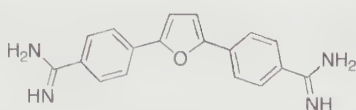
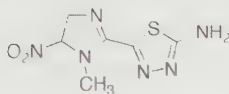
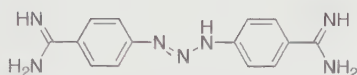

DB289

DB75

megalol

diminazene

Figure 3 Some compounds recently under consideration as new drugs for HAT. DB289 is a prodrug that is converted systemically to yield the diamidine DB75. Megazol has pronounced trypanocidal activity although development was halted due to toxicity associated with the nitroimidazole group. Diminazene is a diamidine used in veterinary medicine.

et al., 2004). Alternative transporters, such as HAPT1 and LATP1, play a less important role in entry of this compound than they do for pentamidine. However, a secondary, minor route of uptake was apparent in experiments following uptake of radiolabelled diminazene into *T. brucei* (de Koning *et al.*, 2004) and indeed knockout cells do retain sensitivity to low micromolar concentrations of the drug, albeit being around 30-fold less sensitive than wild-type cells (Matovu *et al.*, 2003) in the 72-hour Alamar blue assay (Raz *et al.*, 1997). The nature of this secondary route is as yet not certain, although saturation was not reached over the range of drug concentrations tested. Whether another (low affinity) transporter is involved, or whether another route, such as endocytosis plays a role cannot be distinguished on current data. It is also conceivable that diamidines exert a toxic effect at the level of the membrane itself at higher concentrations.

2.4. DB289/DB75

DB289 is an amidoxime prodrug that is converted systemically to its active diamidine derivative, DB75 (Ansede *et al.*, 2004) (Figure 3).

We are currently investigating the uptake profile of this drug, which appears, like diminazene, to depend principally upon the P2 transporter, but with a minor uptake still evident in P2 knockout cells (C. Lanteri, R. Tidwell, M. Stewart and M. Barrett, unpublished).

The situation regarding the DB289 DB75 pairing is of particular interest. DB289 is far less active against trypanosomes than DB75. However, DB75 is active in animals only when given by injection whereas DB289 is active in an oral formulation. The reasons for this relate to the fact that DB289, but not DB75, can cross the intestinal epithelium (Sturk *et al.*, 2004) in quantities sufficient to reach significant levels in blood. DB289 is then metabolised to the active diamidine form by cytochrome P450 and other metabolic enzymes (Ansele *et al.*, 2005). Once systemic, DB75 is then selectively accumulated into trypanosomes via the P2 transporter and it induces death. Uptake of DB289 at the intestinal epithelia appears to take a transcellular route and this compound accumulates across Caco2 monolayers some 75-fold more effectively than DB75 (Sturk *et al.*, 2004). Interestingly, given that DB289 appears to be metabolised significantly during a first pass of the liver, much of the compound has already been converted to DB75 before it reaches the vasculature feeding the brain. It is possible that DB289 could cross the blood-brain barrier more readily than DB75 and the idea that intravenous DB289 could give enhanced activity against late-stage disease has been proposed (Sturk *et al.*, 2004). The extent of blood-brain barrier uptake of diamidine-based trypanocides could, in principle, depend on subtle pharmacokinetic properties, highlighting the importance of the relationship between drug, host and parasite in achieving optimal pharmacological activity (Croft, 1999).

2.5. Suramin

Suramin, a colourless polysulphonated symmetrical naphthalene derivative, was first used against sleeping sickness in 1922 (Voogd *et al.*, 1993). The drug lost favour as a treatment for gambiense sleeping sickness when large numbers of treatment failures were reported in the 1950s although the factors underlying these treatment

failures were never identified with any certainty, and the drug is generally useful for the treatment of the early stage infection due to either *T. b. gambiense* or *T. b. rhodesiense*. The drug has also been considered for a number of other conditions, including some cancers (Voogd *et al.*, 1993) as it interferes with a number of cellular processes.

Poor intestinal absorption, and also a local irritation if given intramuscularly mean that the drug should be administered by slow intravenous injection (Voogd *et al.*, 1993). Dosing at 1 g per week over 6 weeks maintains levels at $150\text{--}200\text{ mg L}^{-1}$. Most of the drug binds to serum proteins. It does not cross the blood brain barrier to levels capable of killing trypanosomes in the CSF at doses given in treatment of stage I disease. Plasma concentrations decline exponentially with a half-life of up to 60 days. About 80% of the dose is eliminated in the urine (Voogd *et al.*, 1993).

Measurements of the rate of uptake into trypanosomes of radio-labelled suramin indicated that rates exceeded those expected for a compound accumulated by fluid phase endocytosis (Fairlamb and Bowman, 1980). Interestingly, recent work by Engstler *et al.* suggests that the rate of fluid-phase endocytosis in bloodstream form African trypanosomes is exceptionally high (Engstler *et al.*, 2004). Marker compounds used to follow uptake revealed that uptake was very rapid, but that most of the marker (90%) was also rapidly released from cells, which, according to their model, gave a net fluid phase uptake rate similar to that estimated by Fairlamb and Bowman. It is not known whether suramin enters via this route, and then, for some reason, fails to be excreted at similarly high rates as the dextran-derivative used as a marker in studying kinetics of endocytosis by Engstler *et al.*

Suramin is highly charged, containing six negative charges at physiological pH and it binds with high avidity to many serum proteins including low-density lipoprotein (LDL) (Vansterkenburg *et al.*, 1993). Trypanosomes have a receptor for LDL (Bastin *et al.*, 1996; Coppens and Courtoy, 2000; Green *et al.*, 2003). Since suramin inhibits LDL uptake (Vansterkenburg *et al.*, 1993) a possible entry route involving the LDL receptor was proposed (Coppens and Courtoy, 2000). However, experiments using mutant trypanosomes (Pal *et al.*, 2002), defective in separate vesicle systems showed that, at

least in procyclic *T. brucei*, overexpression of the vesicle associated G-protein TbRAB4 led to reduction in binding and internalisation of suramin, but not in iodinated LDL. Conversely, cells expressing a permanently activated version of TbRAB5A internalised LDL more quickly than wild-type cells without altering their ability to accumulate suramin. It was concluded that LDL and suramin follow separate pathways into procyclic cells (Pal *et al.*, 2002).

A systematic study of other receptor-mediated routes of uptake into trypanosomes may identify the "suramin receptor" and it remains possible that uptake is via receptor-mediated endocytosis, although it will be of interest to learn more about the role of fluid-phase uptake of suramin and other trypanocidal drugs.

Many hypotheses as to the mode of trypanocidal action have been proposed, but none proven. The drug is highly active against bloodstream forms of the parasite *in vitro* but around a hundredfold less active against procyclic forms of the organisms (Scott *et al.*, 1996), which has led to suggestions that glycolysis (which is essential to bloodstream forms but not procyclics) may be key (Fairlamb and Bowman, 1977, 1980; Wierenga *et al.*, 1987). A number of other pathways too could be targeted by the drug.

Reports on suramin resistance in the field are rare (Barrett, 2003). In animal diseases, however, parasites of the brucei group resistant to this drug have been reported. Laboratory derived lines have also been selected for resistance to suramin over the years (since the 1930s). Several studies have focused on *T. evansi* (Mutugi *et al.*, 1994). One laboratory study pointed to the fact that resistant lines were more difficult to clone and grow than wild-type *T. evansi*. This prompted suggestions that resistance might not be a stable phenotype (Mutugi *et al.*, 1995). However, *T. evansi* isolated from Sudan some 20 years after suramin had been withdrawn from use due to the advent of resistance (El Rayah *et al.*, 1999), were still highly resistant to this drug indicating that resistance can be very stable.

No evidence for a reduction in drug uptake associated with resistance has been reported and mechanisms of resistance are not known. One study did show that a line selected for resistance to melarsen oxide (33-fold resistance) (Scott *et al.*, 1996) had a nearly sixfold decrease in susceptibility to suramin. However, most other studies

have concluded that there is no cross-resistance between suramin and the melamine-based arsenicals or other drugs (Barrett, 2003).

2.6. Eflornithine

Eflornithine, or D,L- α -difluoromethyl ornithine (DFMO), is an analogue of ornithine and acts as a specific suicide inhibitor of the enzyme ornithine decarboxylase (ODC) (Bacchi *et al.*, 1980; Bacchi and Yarlett, 1993). It was first developed as a potential anti-cancer reagent; however, it remains at the trial stage against neoplastic disease (Barrett and Barrett, 2000). The drug also has activity against sleeping sickness caused by *T. b. gambiense*, even in the late stage (Burri and Brun, 2003). A drawback is the drug's lack of activity against rhodesiense sleeping sickness (Iten *et al.*, 1995).

Around half of the dose becomes bioavailable after oral administration (Burri and Brun, 2003). The mean half-life in plasma following intravenous injection is only 3 hours, with 80% of the drug excreted unchanged in urine after 24 hours. In order to be effective against sleeping sickness, the drug needs to be given in large doses. Little of the drug binds to serum proteins. Immediately after a 14-day course, the CSF to plasma ratio is 0.91 in adults and 0.58 in children. Children retain less drug than adults.

Eflornithine inhibits ODC, which is a key enzyme in the biosynthesis of polyamines. The drug has similar affinity for both the mammalian and trypanosomal ODCs. Its specificity against the parasite possibly arises because *T. b. gambiense* ODC is degraded within the cell and replenished at a rate several orders of magnitude slower than its mammalian counterpart (Ghoda *et al.*, 1990).

It has been proposed that lack of activity against rhodesiense parasites relates to the enzyme being more rapidly turned over in that system (Kaminsky *et al.*, 1996). A pulse of DFMO can deprive trypanosomes of ODC and polyamine synthesis for a prolonged period compared with mammalian cells, leading to a cessation of growth. Inhibition of ODC has other results besides a reduction in putrescine and further polyamine biosynthesis. For example it leads to an increase in cellular levels of S-adenosyl methionine which might have

toxic effects (Yarlett and Bacchi, 1988). Inappropriate methylation of proteins, nucleic acids, lipids and other cell components are thus implicated.

Trypanothione levels are also diminished after DFMO treatment (Fairlamb *et al.*, 1987), which might render parasites more vulnerable to oxidative stress. A functional immune system is required to kill the growth-arrested trypanosomes (Bitonti *et al.*, 1986). It has also been reported that *T. brucei* lack polyamine transporters rendering them auxotrophic for polyamines (Fairlamb and Le Quesne, 1997). Conversely, many mammalian cells can scavenge polyamines from plasma using transporters allowing them to bypass the lack of endogenous biosynthesis while *T. brucei* cannot tolerate this situation.

Some early studies in mammalian cells indicated that DFMO uptake was a passive process involving simple diffusion across the membrane (Erwin and Pegg, 1982). It has been reported that uptake of DFMO in *T. brucei* also occurs via passive diffusion across the plasma membrane (Phillips and Wang, 1987; Bellofatto *et al.*, 1987). These observations were based on the fact that uptake appeared to be unsaturable up to the highest DFMO concentration tested and that internal concentration of drug equilibrated with external concentration. However, a saturable process typical of transport-associated uptake in procyclic organisms with a K_m of 244 μM was noted (Phillips and Wang, 1987) and uptake was temperature sensitive. In the yeast *Neurospora crassa*, a basic amino acid transporter has been implicated in uptake of DFMO (since this transporter is lost in strains selected for resistance to the drug) (Davis *et al.*, 1994). Studies in trypanosomes concluded that DFMO does not share an uptake system with ornithine, arginine or lysine. In procyclic cells selected for DFMO resistance, putrescine uptake was noted to be 3–4 times higher than in wild-type lines (Phillips and Wang, 1987). Putrescine at >1 mM allowed parasites to survive DFMO treatment *in vitro* while 0.1 mM did not (Phillips and Wang, 1987). Moreover, *T. brucei* parasites from which the ODC gene had been removed, were also viable and capable of growth provided external putrescine was abundant (Li *et al.*, 1998) (far more abundant than in mammalian serum where it is at around 220 nM). Procyclic cells selected in the laboratory for resistance to DFMO showed reduced

drug accumulation (Phillips and Wang, 1987) although whether this was due to decreased uptake or increased efflux was not determined. These combined data suggest that the mechanism of uptake of DFMO may either be organism (and even life-stage) dependent, or a more complex mechanism of uptake occurs in *T. brucei*. For example, an equilibrative transporter with relatively low affinity for this compound. It would be interesting to investigate this possibility.

The introduction of a different compound with similar inhibitory efficacy against ODC, but which can be accumulated more vigorously by the parasites, might be an excellent drug design strategy.

2.7. Nifurtimox

Nifurtimox was originally licensed for use against South American trypanosomiasis (Stoppani, 1999). The drug contains a nitro group, which is central to its activity. It has also been used in trials, with only limited success (50–80% cure), against *T. b. gambiense* in West Africa (Janssens and Demuynck, 1977; Pépin *et al.*, 1989). However, since it is apparently active against melarsoprol refractory parasites it may still be used, particularly in combinations (Moens *et al.*, 1984; Jennings, 1991), as treatment failures with arsenical monotherapy increase (Brun *et al.*, 2001).

Serum levels are reportedly low when nifurtimox is given orally, peaking 1–3 hours after administration. The drug can accumulate across the blood–brain barrier (Burri *et al.*, 2004). Toxic effects to the central nervous system and peripheral nervous system have been reported.

Nifurtimox is a nitrofuran compound. One electron reduction of the nitro-group generates a potent free radical which may interact with cellular constituents or generate reduced oxygen metabolites believed to cause death of the parasite (Docampo and Moreno, 1986). *N*-acetyl cysteine, an agent that defends against active oxygen species, diminished the effects of nifurtimox against African trypanosomes (Enanga *et al.*, 2003). DNA repair deficient cells, however, were not changed in their sensitivity to this drug (Enanga *et al.*, 2003). The reduction potential of the compound (−260 mV) is such that it is

relatively easily reduced in many cell types. The specificity towards the parasite is thought to be associated with its being more readily reduced by the parasite than the host cells. Moreover, mammalian cells may have better protection against oxidative damage. Specific targets or enzymes capable of reducing the drug cannot be ruled out. An intriguing hypothesis was that trypanothione reductase might be responsible for the reduction (Henderson *et al.*, 1988). Other enzymes that could perform preferential reduction of these compounds in trypanosomes have also been studied (Viode *et al.*, 1999).

Trypanosoma cruzi isolates show various levels of sensitivity to the drug (Murta *et al.*, 1998). There appears to be a correlation between drug uptake and sensitivity (with lines accumulating least drug being least sensitive to it). Uptake of nifurtimox into *T. cruzi* has been reported to occur via passive diffusion across the plasma membrane (Tshako *et al.*, 1991), although studies on uptake have not yet been extended to *T. brucei*.

Interestingly another nitroheterocycle called megazol was proposed to enter trypanosomes via passive diffusion (Barrett *et al.*, 2000). Megazol was equally active against lines of *T. cruzi* showing different sensitivities to nifurtimox (Filardi and Brener, 1987). Another series of melamine-based nitroheterocycles has recently been shown to have good trypanocidal activity (Stewart *et al.*, 2004; Baliani *et al.*, 2005). In spite of their having been designed specifically to enter via the P2 transporter, these latter compounds were of similar activity against wild-type and P2 knockout parasites indicating that alternative uptake routes also exist for these melamine-based compounds.

3. TARGETING OF NOVEL COMPOUNDS TO TRYPANOSOMES VIA PLASMA MEMBRANE TRANSPORTERS

3.1. The P2 Aminopurine Transporter

Two classes of the currently used trypanocidal drugs, the melamino-phenyl arsenicals (e.g. melarsoprol and cymelarsan which are both

converted to melarsen oxide *in vivo*) and the diamidines (e.g. pentamidine, diminazene, DB75) use the P2 aminopurine transporter as a route of entry into *T. brucei*. These trypanosomes contain multiple purine transporters at their plasma membrane. Two adenosine transport systems were defined by Carter and Fairlamb (1993) corroborating earlier accounts of multiple adenosine transporters in these parasites (James and Born, 1980). Since phenylarsen oxide, which is believed to be freely diffusible over lipid bilayers (Scott *et al.*, 1997), was toxic to many cells, while the melamine-linked derivative melarsen oxide is selectively toxic towards trypanosomes, it seemed that the addition of the melamine ring was critical for selective activity, probably by mediating selective uptake. Therefore, Carter and Fairlamb tested the ability of a number of nitrogen-rich compounds (melamine itself being nitrogen rich), when given in excess, to block melarsen oxide induced lysis in trypanosomes that could be detected in a spectrophotometer (Meshnick *et al.*, 1978). Of numerous tested compounds only the 6-aminopurines adenosine and adenine abrogated lysis. It was then shown that at least two adenosine transport systems were operative. One, a general purine nucleoside carrier system that could be measured by following inhibition of adenosine uptake (as a competitive substrate) with inosine (and other nucleosides) was called P1. A second transporter, which appeared specific for 6-aminopurines, adenine and adenosine was called P2. Carter and Fairlamb demonstrated that a parasite line, RU15 (Fairlamb *et al.*, 1992a), that had been selected for resistance to sodium melarsen had lost P2 transport (Carter and Fairlamb, 1993). It was thus concluded that uptake of melaminophenyl arsenicals was mediated by the P2 transporter (Carter and Fairlamb, 1993; Barrett and Fairlamb, 1999; Maser *et al.*, 1999) and that other purine transporter systems were capable of accumulating sufficient quantities of purine to fulfil the parasite's requirement.

A number of other lines that had been selected for resistance to arsenical-based drugs were subsequently shown also to be deficient in the P2 transporter. Using the spectrophotometric lysis assay, Carter and Fairlamb were then able to determine a structure activity profile of compounds capable of inhibiting P2-mediated lysis. The description of this P2 recognition motif (Figure 2) led directly to efforts to

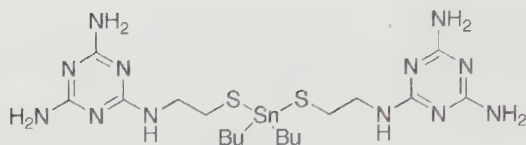
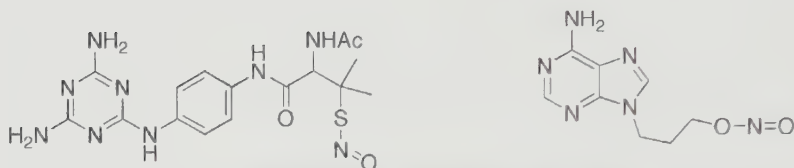
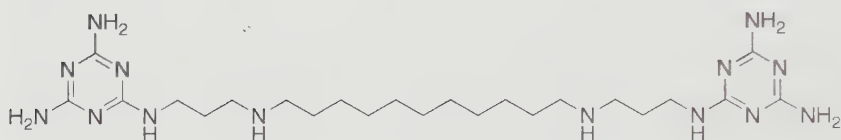
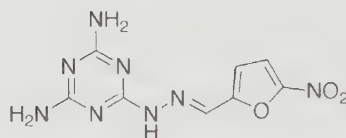
**P2 motif attached to tin derivative****P2 motif attached to NO-releasing moiety****P2 motif attached to polyamine****P2 motif attached to nitrofuran**

Figure 4 A range of compounds with P2 recognition motifs added aiming to enhance selectivity against trypanosomes. These compounds have been designed to enter trypanosome by virtue of their containing a P2 recognition motif (see Section 4.1).

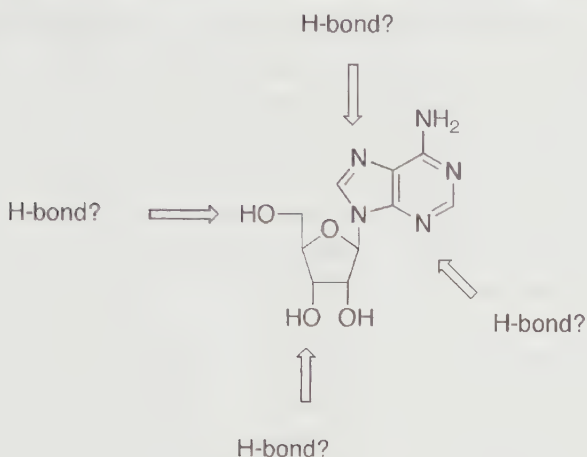
introduce toxins into trypanosomes via this route (Figure 4). For example, potentially toxic tin compounds had been studied by the group of Deleris, and addition of melamine moieties enhanced activity of some members of this class of compounds (Susperregui *et al.*,

1997, 1999). Perie's group were engaged in attempts to produce nitric oxide releasing agents and they too took a P2 delivery strategy (Soulere *et al.*, 1999, 2000). In our own work, polyamine analogues were tagged with melamine moieties (Tye *et al.*, 1998; Klenke *et al.*, 2001) and these did indeed have high affinity for the P2 transporter (assessed by measuring their ability to inhibit adenosine uptake). A number of potently trypanocidal derivatives were identified (Klenke *et al.*, 2001). However, these were also overtly toxic to rodents and further development was halted. Next we went on to tag a number of nitroheterocyclic compounds with melamine moieties too (Stewart *et al.*, 2004; Baliani *et al.*, 2005). This was done since it was already known that trypanosomes are sensitive to nitroheterocyclic compounds including nifurtimox (Vannieuwenhove, 1988), megalol (Enanga *et al.*, 1998, 2003) and a series of other nitro-derivatives (Jennings, 1991; Millet *et al.*, 2002). Genotoxicity has been an issue with the development of nitroheterocycles (Bendesky *et al.*, 2002). Hence we aimed to improve selectivity by delivering compounds to trypanosomes via the P2 transporter. This led to a number of highly active melamine-nitroheterocyclic-based trypanocides (Stewart *et al.*, 2004; Baliani *et al.*, 2005). Interestingly, these compounds were equally active against wild-type and P2 knockout parasites indicating that routes in addition to P2 are probably used in uptake (Stewart *et al.*, 2004; Baliani *et al.*, 2005). This is important since it indicates that such compounds can retain trypanocidal activity even if the P2 transporter is absent. Moreover, we also went on to show that the best lead melamine-nitroheterocycle from our initial experiments was capable of clearing trypanosome infections of mice when given at 20 mg kg (the lowest does tested to date). It will be of great interest to learn the other routes of uptake of these compounds. Maser and Kaminsky too initiated a study specifically looking for compounds carrying the P2 recognition motif (based on their ability to compete with adenosine for the transporter when expressed in yeast) in order to target them to cells and also unearthed a number of interesting trypanotoxins (Maser *et al.*, 2001). The P2 recognition motif has thus become a potent symbol of how transporters can be exploited to carry toxins into cells.

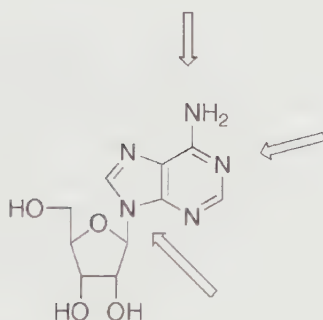
3.2. Other Purine Transporters

In addition to P2, a number of other purine transporters have been identified in trypanosomes using both biochemical and genetic approaches (Carter and Fairlamb, 1993; de Koning and Jarvis, 1998, 1999; de Koning *et al.*, 1998, 2005; Sanchez *et al.*, 1999, 2002, 2004; Carter *et al.*, 2001; Landfear, 2001; Wallace *et al.*, 2002, 2004; Burchmore *et al.*, 2003; Landfear *et al.*, 2004; Natto *et al.*, 2005; Henriques *et al.*, 2003). For example, several nucleobase transporters have been identified. A number of purine antimetabolites enter via the base transporters (Wallace *et al.*, 2002) and an interesting family of trypanocidal tricyclic purines had high affinity for the *T. brucei* H2 hypoxanthine transporter (Wallace *et al.*, 2004). A detailed characterisation of the structure-activity of these has also been performed using inhibition of substrate uptake with analogues to determine which atoms are required for transporter recognition (Wallace *et al.*, 2002). It has been proposed that resistance to the purine antimetabolite allopurinol is difficult to select in *T. brucei* since this compound enters via multiple transporters (Natto *et al.*, 2005). In addition to these studies into purine-base recognition motifs, de Koning and Jarvis also confirmed the P2 recognition motif by measuring the ability of a number of analogues to inhibit adenosine uptake via this route (de Koning and Jarvis, 1999). The same study also revealed the requirement for a ribose moiety for recognition by the P1 component explaining its function as a general nucleoside carrier (Figure 5).

Trypanocidal activity of other purine analogues has been reported by a number of investigators: for example, MDL73811 (Byers *et al.*, 1992), sinefungin (Phelouzat *et al.*, 1995) and 5'-deoxy-5'-(hydroxyethyl)thioadenosine (HETA) (Bacchi *et al.*, 1991). All of these compounds have been proposed to interfere with polyamine metabolism at the level of the methionine cycle, which plays a critical role in regenerating decarboxylated *s*-adenosylmethionine (Berger *et al.*, 1996), the aminopropyl group donor that sustains synthesis of polyamines. The exact routes of uptake of these particular compounds have not been identified, although of interest was the fact that O-acetylation of a number of purine analogues enhanced their activity (Sufrin *et al.*, 1996).



P1 Transporter Interactions



P2 Transporter Interactions

Figure 5 Structure activity profiles for *T. brucei* P1 and P2 purine nucleoside transporter systems. Adenosine enters via the P1 and P2 transporter systems. P2 is encoded by a single gene (*TbAT1*). P1, however, appears to comprise activities of multiple gene products. However, it has been possible to elucidate the structure activity profiles of both systems. Atoms present on adenosine that appear to be important in binding to the transporters are marked by arrows (see de Koning & Jarvis, 1999, for more details).

The situation with regard to purine transporters is complex. The *T. brucei* genome sequence has revealed a multitude of separate genes encoding members of the equilibrative nucleoside transporter (ENT) family (Landfear *et al.*, 2004; de Koning *et al.*, 2005). After the initial

identification of the *TbAT1* gene (Maser *et al.*, 1999) encoding the P2 transporter, and a *TbNT2* gene (Sanchez *et al.*, 1999), which encoded a P1-like activity, a cluster of genes of similar, but non-identical, sequence, related to *TbNT2*, was identified and proposed to encode the P1 component (Sanchez *et al.*, 2002). However, a number of different affinities and substrate specificities were seen for individual members of that cluster of genes related to *TbNT2* (Sanchez *et al.*, 2002). For example, *TbNT5*, 6 and 7 all carried hypoxanthine, while *TbNT2* itself did not.

Some of the *TbNT* cluster were upregulated in the cell line from which the *TbAT1* gene, that encodes P2, had been deleted (Geiser *et al.*, 2005). This indicates some level of cross-regulation in the purine uptake system, which can influence the ability of trypanosomes to carry other purine antimetabolites by other transporters when, for example, *TbAT1* is lost.

It seems likely that further advances in exploiting the whole gamut of purine transporters in *T. brucei* will be made in the future. Importantly, as shown particularly in the case of the P2 transporter, simple purine analogues are not the only type of compounds that may be targeted via these routes, if sufficient information can be learned about the structure–activity relationship of the transporters and their substrates to enable transporter recognition motifs to be tagged on to other compounds.

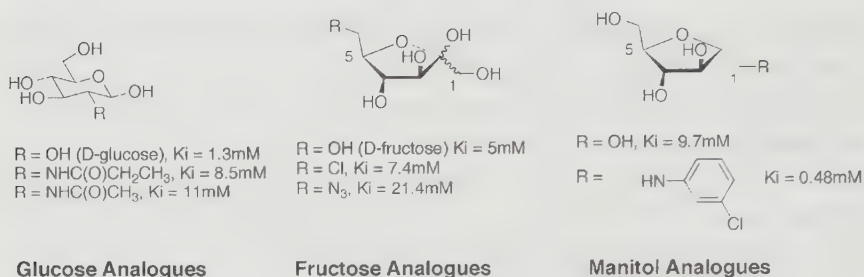
3.3. Hexose Transporters

Bloodstream form trypanosomes depend exclusively on substrate-level phosphorylation through glycolysis to produce ATP (Grant and Fulton, 1956; Oppendoes *et al.*, 1976; Oppendoes and Fairlamb, 1977; Verlinde *et al.*, 2001). Glycolysis is distinctive in trypanosomatids in that the early enzymes of the pathway are found in organelles termed glycosomes (Oppendoes and Borst, 1977; Oppendoes *et al.*, 1977; Oppendoes, 1987). Glucose transport was proposed as the rate-limiting step in glycolytic flux (Terkuile and Oppendoes, 1991). However, this is only true at low external glucose concentrations (Bakker *et al.*, 1999a), and regulation of flux occurs at several other

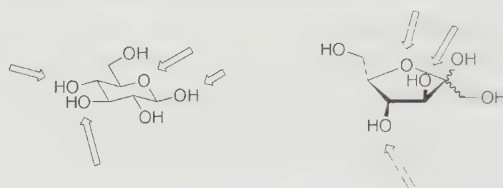
points at physiological (5 mM) concentration of external glucose (Bakker *et al.*, 1999b, 2000).

Glucose transporters have been extensively characterised in several trypanosomatids at the biochemical (Conroy *et al.*, 1987; Eissenthal *et al.*, 1989; Seyfang and Duszenko, 1991, 1993; Fry *et al.*, 1993; Tetaud *et al.*, 1997; Walmsley *et al.*, 1998; Barrett *et al.*, 1998) and molecular genetic (Bringaud and Baltz, 1992, 1993, 1994; Barrett *et al.*, 1995a, 1999; Tetaud *et al.*, 1997; Walmsley *et al.*, 1998) levels. Since many of these parasites have life cycles which take them between insect vectors and mammalian hosts, studying glucose transport in these organisms has shown how different transporters are adapted for different environments. For example, brucei group trypanosomes cycle between the mammalian bloodstream and the tsetse fly gut. *T. brucei* has a pair of transporters. One, THT1 (Bringaud and Baltz, 1992), has a relatively low affinity and high capacity and this isoform operates in bloodstream form organisms that are bathed in high extracellular glucose concentrations. A second transporter, THT2 (Barrett *et al.*, 1995a), has a higher affinity and lower capacity and is the only form operating in procyclic cells that dwell in the insect where glucose is rare. Genes have been cloned for each of these transporters and expressed in heterologous systems, including *Xenopus* oocytes (Bringaud and Baltz, 1993) and also mammalian cells (Barrett *et al.*, 1995a). A detailed structure-activity relationship was determined for THT1 (Eissenthal *et al.*, 1989), which can carry D-fructose (Fry *et al.*, 1993) as well as D-glucose (Figure 6). The transporter can also recognise D-glucose analogues carrying substituents at the C2 and C6 positions (Eissenthal *et al.*, 1989). Reports of an additional glucose transporter gene have also appeared (Bayele *et al.*, 2000), although the predicted structure of the protein encoded by that particular gene might indicate that this protein is not a *bona fide* glucose transporter. Further, hexose transporter gene orthologues have, however, been identified in the *T. brucei* genome but as yet their products have not been functionally characterised.

Following from the derivation of the SAR profile for the transporter (Eissenthal *et al.*, 1989; Fry *et al.*, 1993), further studies showed that for D-glucose there is a limit to substituents which can be tolerated at the C6 position (Azema *et al.*, 2004). This probably relates



Compounds which interact with the glucose transporter



Putative sites for H-bonding interactions with receptor for D-glucose and D-fructose

Figure 6 Structure–activity profiles for the *T. brucei* hexose transporter. A number of carbohydrates and their analogues that interact with the *T. brucei* hexose transporter THT1 (Azema *et al.*, 2004) are shown in the top part of the figure. Those atoms present in glucose and fructose believed to interact with the transporter are marked with arrows in the lower part of the figure (see Eienthal *et al.*, 1989 and Fry *et al.*, 1993 for more details).

to the orientation of sugar binding such that large groups sterically hinder binding at the exofacial binding site.

To test directly whether D-glucose analogues substituted at the C2 position could actually enter the parasites, the uptake of D-glucosamine (amino-group substituting for the hydroxyl group at position 2) and also *N*-acetyl-D-glucosamine where a methylated carbonyl group is added to this site were measured (Azema *et al.*, 2004). It was shown that substituents at the C2 position can be tolerated by the exofacial binding site, but that a step in the THT1-mediated transport pathway, subsequent to binding at the exofacial site, cannot operate when using *N*-acetyl-D-glucosamine. These data suggest that while binding at the exofacial site is still possible, analogues with

bulky groups at position C2 are unlikely to actually enter via this pathway.

D-Fructose analogues, including those based on 2,5-anhydro-D-mannitol, were also shown to interact with the transporter (Fry *et al.*, 1993; Azema *et al.*, 2000, 2004) and large, aromatic groups added to C1 in some cases produced compounds with higher apparent affinity than D-fructose itself. It seems unlikely that these compounds would be translocated if similar size restrictions to those for *N*-acetyl-D-glucosamine uptake are applied to the D-fructose analogues. Flux modelling of glucose catabolism (Bakker *et al.*, 1999a, b, 2000) indicated that inhibition of the glucose transporter itself would be an effective way of slowing glycolysis to lethal levels in bloodstream form trypanosomes. Promising results were obtained with hexose analogues carrying alkylating halogen groups at permissible positions (Azema *et al.*, 2004). The size of the compounds indicates that they are unlikely to be internalised via the transporter thus the toxic effects probably relate to covalent bonding between the activated carbon centre on the inhibitor and a nucleophilic residue on the transporter itself. These halogenated hexose analogues are unlikely to be specific for trypanosome transporters but they do demonstrate that specific inhibitors of the transporter could be useful in trypanosomiasis chemotherapy. Several inhibitors of mammalian GLUT1 (e.g. cytochalasin B and phloretin) have an activity towards the trypanosome hexose transporter (Seyfang and Duszenko, 1991) that is several orders of magnitude less than for GLUT1. This demonstrates that structural and functional differences between mammalian and trypanosomal transporters can permit selective inhibition of the trypanosomal transporter (Walmsley *et al.*, 1998; Barrett *et al.*, 1999). Several triazene-based dyes, e.g. Cibacron blue were also shown to have inhibitory activity against the *T. brucei* glucose transporter (Bayele, 2001).

Perhaps because of the high concentration of glucose present in plasma, and the relatively stringent SAR profile for this transporter, the hexose transporter may be less useful than the aforementioned purine transporters as a conduit to carry new drugs into these parasites. The transporter itself, however, would appear to be a good target.

3.4. Pyruvate and Glycerol Efflux Systems

The simplified glycolytic scheme in bloodstream form trypanosomes ends with the production of pyruvate that is secreted from cells. An extension of the flux analysis that identified the glucose transporter as a good drug target also predicted that inhibition of pyruvate efflux would be an effective way of killing bloodstream form trypanosomes (Eisenthal and Cornish-Bowden, 1998). Bloodstream form trypanosomes are relatively inefficient at controlling their internal pH (Thissen and Wang, 1991; Nolan *et al.*, 2000; Vanderheyden *et al.*, 2000) and it has been shown that they are vulnerable to relatively minor changes in extracellular pH (Nolan *et al.*, 2000). Pyruvate efflux (and uptake) has been proposed to occur using a facilitative diffusion transporter at the parasite's plasma membrane (Wiemer *et al.*, 1992, 1995; Barnard *et al.*, 1993; Barnard and Pedersen, 1994;), although it has also been proposed to occur in a proton-coupled process (Vanderheyden *et al.*, 2000) that itself plays a key role in regulating pH. Inhibitors of pyruvate transport, including UK5099 (Wiemer *et al.*, 1995) were shown to inhibit the transporter. They also caused pyruvate to accumulate inside cells, which led to acidification, inhibition of glycolysis and cell death. Development of specific substrates for the pyruvate efflux protein would offer an alternative route for targeting new drugs into trypanosomes. For example, a number of other monocarboxylate-derived molecules could be targeted into cells through this route.

Under anaerobic conditions, bloodstream form trypanosomes continue to produce ATP through glycolysis without using the alternative oxidase system to regenerate NAD for sustained activity of the pathway. Instead, glycerol 3-phosphate is converted to glycerol through the reversed action of glycerol kinase (Opperdoes and Fairlamb, 1977; Fairlamb *et al.*, 1977; Kralova *et al.*, 2000; Steinborn *et al.*, 2000). This reaction can be prevented if glycerol levels rise to a degree that inhibits the reversed reaction through mass action (Opperdoes and Fairlamb, 1977). Accordingly, the parasites also require an effective means of removing glycerol from the cell. An equilibrative plasma membrane transporter for glycerol was characterised (Wille *et al.*, 1998) and subsequently three genes encoding aquaglyceroporins (TbAQP1-3) were identified in the *T. brucei* genome (Uzcategui *et al.*, 2004). All

three were shown to be capable of excreting accumulated glycerol when expressed in yeast. Moreover, the three had somewhat different properties with regard to substrate specificity and also expression profiles. TbAQP 3 is also capable of carrying ribitol and erythritol and this form is expressed in long, slender bloodstream forms (Uzcategui *et al.*, 2004). To date, the aquaglyceroporins in *T. brucei* have not been specifically exploited to carry drugs. However, in *Leishmania donovani*, a single aquaglyceroporin (related to a family of authentic aquaporins) (Gourbal *et al.*, 2004; Beitz, 2005) has been implicated in the uptake of trivalent antimony, the active component of pentostam, a drug of choice still used in leishmaniasis chemotherapy. Over-expression of the LmAQP1 gene in *Leishmania major* led to substantial increases in sensitivity to metalloids antimony and arsenic-based drugs (Gourbal *et al.*, 2004; Beitz, 2005). Moreover, loss of just a single allele of the gene led to a 10-fold reduction in sensitivity to these drugs. Aquaglyceroporins have been implicated in the uptake of metalloids in several systems (Liu *et al.*, 2004; Beitz, 2005) thus it will be of interest to determine whether toxins can also be targeted to trypanosomes through their aquaglyceroporins. Trypanosomes are sensitive to hydroxyurea (Mutomba and Wang, 1996), a known substrate of aquaglyceroporin in other systems.

3.5. Amino Acid Transporters

A family of around 40 genes encoding amino acid transporters is present in the *T. brucei* genome making it the largest, and most diverse, family of transporters encoded in the trypanosome genome (Berriman *et al.*, 2005). Efforts are under way to characterise each of these genes in a systematic way. Similar work is also under way in *Leishmania* (Akerman *et al.*, 2004) and *T. cruzi* (Bouvier *et al.*, 2004). Based on experience with a similarly diverse array of purine transporters it seems probable that among the amino acid transporters some may present substrate recognition profiles that will enable design of toxic molecules that may selectively enter trypanosomes via these portals. Some amino acid transporters fulfil similar criteria to purine transporters such as the P2 transporter with regard to their transport

characteristics. In some cases, the transporters' natural substrates are of low plasma abundance, so these transporters should have correspondingly high affinities for their substrate (and hence maybe also substrate analogues). A drug using such a carrier will also have relatively low competition from natural substrate. Chemical complexity of the substrate is also important. The more potentially reactive, diverse chemical groups on a substrate, the higher the chance that a trypanosome transporter will recognise different moieties than the cognate mammalian ones. Amino acids possess a plethora of potentially reactive motifs. Active, concentrative transport can also ensure that compounds continue to enter at high rates regardless of transcellular concentration gradients, again important in concentrating drugs within the cells. Many of the purine transporters appear to utilise a proton motif force (pmf), which comprises the gradient of protons across the membrane plus the electrochemical gradient established by other ionic species, to accumulate within trypanosomes (de Koning *et al.*, 1998; de Koning, 2001a) and some amino acids also appear to use the proton motif force (Hasne and Barrett, 2000b).

A number of amino acid analogues are known to be toxic to trypanosomes (Figure 7). Eflornithine itself is an amino acid analogue and as we noted above there is a possibility that its uptake is transporter mediated. Other amino acid analogues with documented trypanocidal activity include buthionine sulfoximine (BSO) (Arrick *et al.*, 1981; Enanga *et al.*, 2003; Huynh *et al.*, 2003), azaserine (Jaffe, 1963), 6-diazo-5-oxo-norleucine (DON) (Hofer *et al.*, 2001) and acivicin (Hofer *et al.*, 2001), and several of its analogues (all of which have also been shown to be effective antimetabolites used against some cancerous cell types (Ahluwalia *et al.*, 1990)). Catecholamine and indolealkylamine derivatives of aromatic amino acids (Owolabi *et al.*, 1990), e.g. 5-hydroxydopamine, 6-hydroxydopamine, 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine were also shown to be potently active *in vivo* and also *in vitro* when tested on parasites grown over a monolayer of mammalian cells. The necessity for such a feeder layer when these assays were performed makes it difficult to ascertain whether the aromatic amino acid derivatives themselves, or metabolic products, e.g. oxidized forms, were responsible for the activity. The pronounced trypanocidal activity of these compounds,

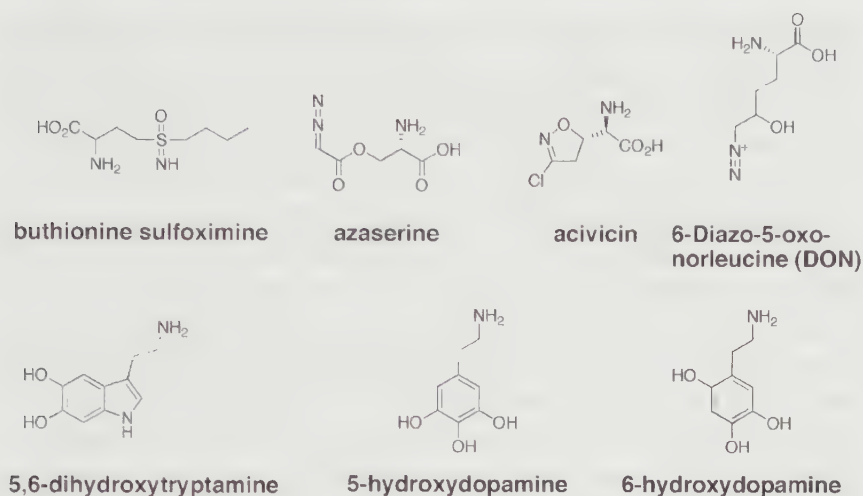


Figure 7 Amino acid analogues with known trypanocidal activity. A number of amino acid analogues with pronounced trypanocidal activity have been identified (see Section 4.5).

coupled to the fact that aromatic amino acid analogues have been considered as important compounds in mammalian pharmacology, and neuropharmacology in particular, would indicate that the roles of aromatic amino acids in trypanosomes and the potential trypanocidal activity of aromatic amino acid analogues should be systematically studied. Aromatic amino acids do appear to have an important role in the methionine cycle that plays a key role in polyamine biosynthesis (Berger *et al.*, 1996). Early work also suggested that aromatic amino acid metabolism in African trypanosomes might generate important metabolic biomarkers of use in diagnosis (Hall and Seed, 1981, 1984).

Some thiazine-linked amino acids showed good *in vitro* activity against *T. brucei* and also led to temporary reduction of parasitaemia in mice (Vanbogaert *et al.*, 1993). These compounds had been designed as putative inhibitors of trypanothione biosynthesis, although it appears that this was not their mode of action, which remains uncertain.

Proline transport in procyclic trypanosomes has been studied in detail, since this nutrient is the principal source of carbon and energy

in the form of the parasite which proliferates inside the midgut of the tsetse fly vector (L'hostis *et al.*, 1993; Lamour *et al.*, 2005). Early studies (Jaffe *et al.*, 1969; Voorheis, 1971, 1973, 1977; Owen and Voorheis, 1976; Hansen, 1979) indicated that a family of amino acid transporters with overlapping substrate specificities are operative in trypanosomes, as in most other organisms. Methionine transport was also studied (Hasne and Barrett, 2000b) and in this instance a specific relatively high-affinity transporter was identified. Since methionine uptake is essential to *T. brucei* (Schmidt *et al.*, 2002), this portal would be a good route to introduce toxins into cells as the transporter should, presumably, be essential and thus its loss not possible.

Interestingly, in *L. major*, a relative of the amino acid transporter family, LmPOT1, was identified and shown to carry the polyamines putrescine and spermidine (Hasne and Ullman, 2005). The *Leishmania* gene was also functionally expressed in *T. brucei*, which itself may be deficient in designated polyamine transporters (Fairlamb and Le Quesne, 1997). The hydrophilic N and C termini that appear to define the *Leishmania* LmPOT1 polyamine transporter protein can identify homologues in *T. cruzi* (which have measurable high affinity polyamine uptake (LeQuesne and Fairlamb, 1996), but not in *T. brucei*. A lack of polyamine transporters might contribute to the sensitivity of *T. brucei* to efloornithine, since they cannot accumulate sufficient polyamines from plasma (where they are present in low abundance) to bypass inhibition of synthesis. External putrescine at concentrations greater than 50 μM can protect trypanosomes against the action of efloornithine, and also permit conditional growth of parasites from which ODC is knocked out (Li *et al.*, 1998). This indicates that putrescine will enter cells, although whether this involves a designated polyamine transporter, or entry through alternative routes when provided at non-physiological levels is not certain.

3.6. Folate/Biopterin Transporters

Antifolates have been important components of antimicrobial chemotherapy and several drugs used against apicomplexan parasites including *Plasmodium* and *Toxoplasma* belong to this pharmaceutical

class (Hyde, 2002). Antifolates have had less success against trypanosomatids, although a large body of evidence relating to their activity against *Leishmania* in particular has accumulated (Hardy *et al.*, 1997; Ouellette *et al.*, 2002). Studies into laboratory-selected resistance to these drugs in *Leishmania* have revealed a number of interesting features related to the biochemistry of folate and pterins in these organisms. Folate and biopterin (Figure 8) transporters, which in *L. major* have been shown to be encoded by a family of at least 14 genes of the BT (biopterin transporter) family, are implicated in drug uptake (Lemley *et al.*, 1999; Cunningham and Beverley, 2001; Richard *et al.*, 2002, 2004; Papadopoulou *et al.*, 2002; El Fadili *et al.*, 2004).

A gene, originally termed *ORFG*, found at the LD1 locus in *L. donovani*, that was frequently amplified in selection of drug resistance, was shown to encode a biopterin transporter and termed BT1 (Lemley *et al.*, 1999). Moreover, loss of some transporters can underlie resistance to antifolates. For example, the antifolate drug methotrexate appears to be selectively accumulated by folate transporters in *Leishmania*. The principal folate transporter in *L. infantum* appears to be FT1 (Richard *et al.*, 2004), with an affinity for folate of $0.4 \mu\text{M}$. Another member of the family, FT5, in *L. tarentolae*, has a lower capacity but higher affinity ($0.08 \mu\text{M}$ (Richard *et al.*, 2002)) for folate. Methotrexate also enters through one or more members of the family. In one particular *L. tarentolae* line selected for resistance to methotrexate, drug transport was reduced, but a separate biopterin transporter was found to be overexpressed (Kundig *et al.*, 1999). The

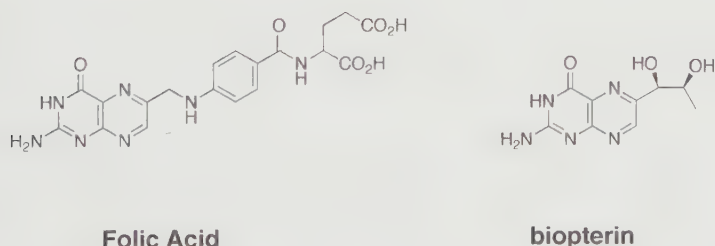


Figure 8 Folic acid and biopterin structures. Trypanosomes, like *Leishmania*, have highly active transporters for folate and biopterin, the structures of which are shown here.

reason for this appears to be that the overexpressed biopterin transporter allows low-capacity uptake of folate but not methotrexate. Thus folate-transporter defective cells overexpressing the pterin transporter can carry sufficient folate to fulfil cellular need, while simultaneously diminishing uptake of methotrexate. In *T. brucei*, clear homologues of the *Leishmania* folate/pterin transporters is encoded by genes of three closely related families. A cluster of genes most closely related to the *Leishmania* biopterin transporters is found on chromosome 1, a cluster most closely related to *Leishmania* folate transporters is on chromosome 8, with the expression site associated gene, *ESAG10* (Gottesdiener, 1994), also belonging to this family. Direct evidence that any of these are folate biopterin transporters is yet to be published, although we have shown both folate and biopterin to be carried into *T. brucei* (unpublished observations).

A number of diaminopyrimidines (Chowdhury *et al.*, 1999, 2001; Pez *et al.*, 2003; Khabnadideh *et al.*, 2005) were shown to have trypanocidal activity, and these were designed and shown to effectively inhibit dihydrofolate reductase in *T. brucei* or other trypanosomatids. We have shown that some of these (at least) have binding affinity for the P2 transporter; however, against the P2-transporter knockout cells they retained activity (unpublished data). The folate biopterin transporter family offers reasonable candidates as being responsible for uptake of these diaminopyrimidines.

3.7. Lipid Transporters

Lipid transporters have recently been shown to be critical in the uptake of ether lipid analogues including the orally available drug miltefosine in *Leishmania* (Perez-Victoria *et al.*, 2001, 2003a, b; Seifert *et al.*, 2003). *Leishmania* resistant to miltefosine were initially shown to have reduced ability to carry the drug into the cells (Perez-Victoria *et al.*, 2003a). Moreover, uptake of a number of fluorescent lipid analogues was also noted. Functional cloning revealed that an ABC (ATP-binding cassette) type protein is involved in phospholipid (and miltefosine) uptake in these cells and its mutation underlies resistance (Perez-Victoria *et al.*, 2003b).

T. brucei has been shown to accumulate lipids. It has been widely perceived that uptake of lipids bound to lipoproteins has been central to lipid uptake. However, it is clear that lysophospholipids are accumulated rapidly by trypanosomes (Samad *et al.*, 1988; Mellors and Samad, 1989; Bowes *et al.*, 1993), as are some fatty acids (Voorheis, 1980). *T. brucei* also has clear homologues of the *Leishmania* P-glycoprotein-type lipid transporter gene involved in miltefosine uptake. Interestingly, however, *T. brucei* are substantially less sensitive to miltefosine than *Leishmania* (Croft *et al.*, 2003), and to date functional analyses of putative lipid transporter genes in *T. brucei* have not been performed.

A number of other studies have aimed at improving targeting of toxins to trypanosomes by conjugating them to lipids. In addition to the possibility of specific transport-driven uptake, lipid conjugates might enter at an increased rate through passive diffusion or other lipid-based uptake mechanisms for instance as components of serum-lipoprotein complexes. Tin derivatives (Shuaibu *et al.*, 2003), and also derivatives of DFMO (Loiseau *et al.*, 1998), were tested for trypanocidal activity and shown to have properties that distinguished them from their parent compounds.

4. DELIVERY OF DRUG CONJUGATES AND ENCAPSULATED FORMS

Some advances in understanding about drug uptake in *Leishmania* have preceded understanding of drug uptake in trypanosomes. This is clearly illustrated by the examples of aquaglyceroporins, pterin and lipid uptake outlined above. *Leishmania* also serve as a good model to illustrate the potential of improving drug delivery via encapsulating drugs into vesicular liposomal or other lipid-type complexes (Yardley and Croft, 2000). Uptake of liposomes is not transporter mediated, but small particles can be carried into cells through fluid-phase endocytosis, or receptor-mediated endocytosis, provided suitable receptors exist. In the case of amphotericin B, the encapsulation of drug into liposomes (Ambisome) has greatly reduced the host-toxicity of the drug while retaining leishmanicidal activity (Yardley and Croft,

2000). There are several possible explanations as to why efficacy improves when amphotericin B is encapsulated. The drug may be released slowly, increasing retention and exposure time to parasites and at the same time reducing the amount of drug being delivered to host cells. Another suggestion relates to the fact that *Leishmania* dwell within macrophages, and these cells also engulf liposomes, thus increasing selective delivery of drug to intracellular amastigotes. The biggest advantage enjoyed by encapsulating amphotericin B is the decrease in host-toxicity of the drug. Given that melarsoprol, in particular, causes frequent serious, often lethal, side-effects, it is interesting to note that melarsoprol has been formulated in microparticles (Gibaud *et al.*, 2002). To date, studies into the efficacy or safety of the microparticulated form of the drug have not been reported but it would be of interest to determine if host-toxicity is reduced without loss of trypanocidal activity. Extraordinarily, melarsoprol can also be applied topically and still achieve cure of trypanosomiasis with greatly reduced incidence of toxicity in mice (Jennings *et al.*, 1993; Atouguia *et al.*, 1995). Diminazine aceturate (Berenil) (Olbrich *et al.*, 2004) has also been formulated in lipid-drug nanoparticles which retain trypanocidal activity *in vitro*. Whether this improves activity *in vivo* is yet to be reported.

As described above, published evidence suggests that suramin may enter trypanosomes via receptor-mediated endocytosis, although the identity of those receptors remains to be convincingly demonstrated, and fluid-phase endocytosis cannot be ruled out. Trypanosome lytic factor (apolipoprotein L1; apoL1) clearly enters trypanosomes via receptor-mediated endocytosis (Vanhamme *et al.*, 2003; Perez-Morga *et al.*, 2005). Interestingly *T. b. rhodesiense* is not susceptible to TLF because the serum resistance associated *sra* gene product (Degreef *et al.*, 1992; Degreef and Hamers, 1994; Xong *et al.*, 1998) somehow interferes with the trypanocidal activity of this protein, that is believed to perforate lysosomal membranes (Perez-Morga *et al.*, 2005). Notwithstanding, apoL1 does appear to be internalised even in serum-resistant trypanosomes and this could be used as a vehicle to which drugs are coupled. The one receptor that has been characterised at the molecular level is the transferrin receptor that plays a critical role in iron accumulation in trypanosomes. The receptor is

encoded by a pair of genes, *ESAG* 6 and *ESAG* 7, and it has been characterised in some detail (Schell *et al.*, 1991a, b; Steverding *et al.*, 1994, 1995; Bitter *et al.*, 1998; Gerrits *et al.*, 2002; Mussmann *et al.*, 2003, 2004).

Conjugating toxins to transferrin might also represent a good way of introducing drugs to cells (although the apparent capacity of trypanosomes to express different isoforms of the transferrin receptor to enable recognition of different isoforms of transferrin in various mammalian systems might indicate that specific transferrin would be required for optimal uptake in different hosts). Nok and Nok conjugated azanthraquinone to bovine transferrin and in so doing improved trypanocidal activity (Nok and Nock, 2002). Activity might have been further enhanced if they used a trypanosome adapted to expression of a transferrin receptor isoform that was optimally adapted to the bovine form used in the conjugation. It would be of interest to determine whether selective activity of other compounds could be induced by coupling to transferrin, LDL or other substrates, even suramin, that enter trypanosomes through receptor-mediated endocytosis.

5. EFFLUX PUMPS

In addition to their ability to carry drugs into cells, it is also clear that *T. brucei*, like other cells, possesses a plethora of pumps capable of excreting xenobiotics as well as unwanted end products of metabolism. To date, relatively little has been shown with regard to the role of such efflux pumps in drug resistance and no instances where over-expression of such pumps (or their mutation) during selection of drug resistance have been reported (Maser and Kaminsky, 1998; Maser *et al.*, 2003). This is unlike the situation in *Leishmania* where over-expression of the multidrug resistance like protein (MDR1) and *P*-glycoprotein (e.g. PGPA) are frequently associated with resistance to drugs (Legare *et al.*, 2001; Ouellette *et al.*, 2001; Leandro and Campino, 2003). The completion of the *T. brucei* genome sequence has revealed multiple potential efflux pumps, although systematic studies to identify cellular localisation (e.g., at the plasma membrane

or lining vesicles into which xenobiotics may be pumped prior to release from the cell) have not been performed for most. The best studied of the efflux pump proteins is the multidrug resistance associated protein TbMRPA. Overexpression of an ectopic copy of the gene leads to resistance to melarsoprol (Shahi *et al.*, 2002). Moreover, knockdown of this gene, by RNA interference, reveals that cells become hypersensitive to melarsoprol (Alibu *et al.*, 2006). The pump is located at the plasma membrane and is thought to carry xenobiotics conjugated to trypanothione. This could be supportive of trypanothione having a role in the mode of action of melarsoprol. The related transporter TbMRPE does not appear to have a similar function. It will be of interest to systematically identify roles for each of the putative efflux pump molecules using gene overexpression and knockout, or RNAi-induced knockdown in *T. brucei*.

6. CONCLUSION

Trypanosoma brucei has a variety of different plasma membrane transporters, several of which have potential for the delivery of trypanocidal agents. The P2 aminopurine transporter turns out to be a significant route of entry for two trypanocidal drugs for human disease (melarsoprol and pentamidine) and diminazene, which is used for animal trypanosomiasis. The uptake of these molecules is complex and several other transporters also play varying roles in the uptake of each drug. The relative contribution of different transporters depends on the structure of the substrate, and relatively small structural changes can have a big effect on routes of uptake. However, the melamine and benzamidine motifs are substrates for the P2 transporter and/or related transporters. Attaching trypanocidal agents to these "P2" recognition motifs appears to be an effective means of selectively delivering agents to trypanosomes.

The purine transporters are of particular interest for drug delivery as they have evolved to show high affinity for their substrates, a necessity arising from the relatively low plasma levels of purines. Uptake through these transporters is active, employing the proton motif force to accumulate against a concentration gradient. Various

other trypanosome transporters have also been investigated. Amino acid transporters also offer good potential as conduits by which to deliver new drugs. Indeed eflornithine and other trypanocidal amino acid analogues could potentially enter through amino acid transporters. Glucose transporters have been well characterised in trypanosomes and structure activity relationship studies undertaken with analogues of glucose and fructose, to determine features required for molecular recognition. There are numerous other trypanosome transporters, which are still to be thoroughly investigated, and these may offer additional methods for selectively targeting trypanocidal agents to the trypanosomes. The number of transporters present in the *T. brucei* membrane, as predicted through genome sequence information, indicates that a systematic study of these will yield a multitude of routes by which toxic compounds can be selectively directed into the interior of trypanosomes.

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REFERENCES

- Agbe, A. and Yielding, K.L. (1995). Kinetoplasts play an important role in the drug responses of *Trypanosoma brucei*. *Journal of Parasitology* **81**, 968–973.
- Ahluwalia, G.S., Grem, J.L., Hao, Z. and Cooney, D.A. (1990). Metabolism and action of amino-acid analog anticancer agents. *Pharmacology & Therapeutics* **46**, 243–271.
- Akerman, M., Shaked-Mishan, P., Mazareb, S., Volpin, H. and Zilberstein, D. (2004). Novel motifs in amino acid permease genes from *Leishmania*. *Biochemical and Biophysical Research Communications* **325**, 353–366.
- Alibu, V.P., Richter, C., Voncken, F., Marti, G., Shahi, S., Renggli, C.K., Seebeck, T., Brun, R. and Clayton, C. (2006). The role of *Trypanosoma brucei* MRPA in melarsoprol susceptibility. *Molecular and Biochemical Parasitology* **146**, 38–44.

- Allsopp, R. (2001). Options for vector control against trypanosomiasis in Africa. *Trends in Parasitology* **17**, 15–19.
- Ansele, J.H., Anbazhagan, M., Brun, R., Easterbrook, J.D., Hall, J.E. and Boykin, D.W. (2004). *O*-Alkoxyamidine prodrugs of furamidine: *in vitro* transport and microsomal metabolism as indicators of *in vivo* efficacy in a mouse model of *Trypanosoma brucei rhodesiense* infection. *Journal of Medicinal Chemistry* **47**, 4335–4338.
- Ansele, J.H., Voyksner, R.D., Ismail, M.A., Boykin, D.W., Tidwell, R.R. and Hall, J.E. (2005). *In vitro* metabolism of an orally active *O*-methyl amidoxime prodrug for the treatment of CNS trypanosomiasis. *Xenobiotica* **35**, 211–226.
- Arrick, B.A., Griffith, O.W. and Cerami, A. (1981). Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. *Journal of Experimental Medicine* **153**, 720–725.
- Atougua, J.M., Jennings, F.W. and Murray, M. (1995). Successful treatment of experimental murine *Trypanosoma brucei* infection with topical melarsoprol gel. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89**, 531–533.
- Azema, L., Bringaud, F., Blonski, C. and Perie, J. (2000). Chemical and enzymatic synthesis of fructose analogues as probes for import studies by the hexose transporter in parasites. *Bioorganic & Medicinal Chemistry* **8**, 717–722.
- Azema, L., Claustre, S., Alric, I., Blonski, C., Willson, M., Perie, J., Baltz, T., Tetaud, E., Bringaud, F., Cotten, D., Opperdoes, F.R. and Barrett, M.P. (2004). Interaction of substituted hexose analogues with the *Trypanosoma brucei* hexose transporter. *Biochemical Pharmacology* **67**, 459–467.
- Bacchi, C.J. and Yarlett, N. (1993). Effects of antagonists of polyamine metabolism on African trypanosomes. *Acta Tropica* **54**, 225–236.
- Bacchi, C.J., Nathan, H.C. and Hutner, S.H. (1980). Polyamine metabolism – a potential therapeutic target in trypanosomes. *Science* **210**, 332–334.
- Bacchi, C.J., Sufrin, J.R., Nathan, H.C., Spiess, A.J., Hannan, T., Garofalo, J., Alecia, K., Katz, L. and Yarlett, N. (1991). 5'-Alkyl-substituted analogs of 5'-methylthioadenosine as trypanocides. *Antimicrobial Agents and Chemotherapy* **35**, 1315–1320.
- Bakker, B.M., Michels, P.A.M., Opperdoes, F.R. and Westerhoff, H.V. (1999a). What controls glycolysis in bloodstream form *Trypanosoma brucei*? *Journal of Biological Chemistry* **274**, 14551–14559.
- Bakker, B.M., Walsh, M.C., ter Kuile, B.H., Mensonides, F.I.C., Michels, P.A.M., Opperdoes, F.R. and Westerhoff, H.V. (1999b). Contribution of glucose transport to the control of the glycolytic flux in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 10098–10103.
- Bakker, B.M., Westerhoff, H.V., Opperdoes, F.R. and Michels, P.A.M. (2000). Metabolic control analysis of glycolysis in trypanosomes as an

- approach to improve selectivity and effectiveness of drugs. *Molecular and Biochemical Parasitology* **106**, 1–10.
- Baliani, A., Bueno, G.J., Stewart, M.L., Yardley, V., Brun, R., Barrett, M.P. and Gilbert, I.H. (2005). Design and synthesis of a series of melamine-based nitroheterocycles with activity against trypanosomatid parasites. *Journal of Medicinal Chemistry* **48**, 5570–5579.
- Barnard, J.P. and Pedersen, P.L. (1994). Alteration of pyruvate metabolism in African trypanosomes during differentiation from blood-stream into insect forms. *Archives of Biochemistry and Biophysics* **313**, 77–82.
- Barnard, J.P., Reynafarje, B. and Pedersen, P.L. (1993). Glucose catabolism in African trypanosomes – evidence that the terminal step is catalyzed by a pyruvate transporter capable of facilitating uptake of toxic analogs. *Journal of Biological Chemistry* **268**, 3654–3661.
- Barrett, M.P. (2000). Problems for the chemotherapy of human African trypanosomiasis. *Current Opinion in Infectious Diseases* **13**, 647–651.
- Barrett, M.P. (2001). Veterinary link to drug resistance in human African trypanosomiasis? *Lancet* **358**, 603–604.
- Barrett, M. P. (2003). *Drug resistance in sleeping sickness*. WHO Expert Committee on African trypanosomiasis (sleeping sickness), pp. 96–111.
- Barrett, S.V. and Barrett, M.F. (2000). Anti-sleeping sickness drugs and cancer chemotherapy. *Parasitology Today* **16**, 7–9.
- Barrett, M.P. and Fairlamb, A.H. (1999). The biochemical basis of arsenical-diamidine crossresistance in African trypanosomes. *Parasitology Today* **15**, 136–140.
- Barrett, M.P., Tetaud, E., Seyfang, A., Bringaud, F. and Baltz, T. (1995a). Functional expression and characterization of the *Trypanosoma brucei* procyclic glucose transporter, THT2. *Biochemical Journal* **312**, 687–691.
- Barrett, M.P., Zhang, Z.Q., Denise, H., Giroud, C. and Baltz, T. (1995b). A diamidine-resistant *Trypanosoma equiperdum* clone contains a P2 purine transporter with reduced substrate affinity. *Molecular and Biochemical Parasitology* **73**, 223–229.
- Barrett, M.P., Tetaud, E., Seyfang, A., Bringaud, F. and Baltz, T. (1998). Trypanosome glucose transporters. *Molecular and Biochemical Parasitology* **91**, 195–205.
- Barrett, M.P., Walmsley, A.R. and Gould, G.W. (1999). Structure and function of facilitative sugar transporters. *Current Opinion in Cell Biology* **11**, 496–502.
- Barrett, M.P., Fairlamb, A.H., Rousseau, B., Chauviere, G. and Perie, J. (2000). Uptake of the nitroimidazole drug meglumine by African trypanosomes. *Biochemical Pharmacology* **59**, 615–620.
- Barrett, M.P., Burchmore, R.J.S., Stich, A., Lazzari, J.O., Frasch, A.C., Cazzulo, J.J. and Krishna, S. (2003). The trypanosomiasis. *Lancet* **362**, 1469–1480.

- Bastin, P., Stephan, A., Raper, J., SaintRemy, J.M., Opperdoes, F.R. and Courttoy, P.J. (1996). An M_r 145000 low-density lipoprotein (LDL)-binding protein is conserved throughout the Kinetoplastida order. *Molecular and Biochemical Parasitology* **76**, 43–56.
- Bayele, H.K., Eissenthal, R.S. and Towner, P. (2000). Complementation of a glucose transporter mutant of *Schizosaccharomyces pombe* by a novel *Trypanosoma brucei* gene. *Journal of Biological Chemistry* **275**, 14217–14222.
- Bayele, H.K. (2001). Triazinyl derivatives that are potent inhibitors of glucose transport in *Trypanosoma brucei*. *Parasitology Research* **87**, 911–914.
- Beitz, E. (2005). Aquaporins from pathogenic protozoan parasites: structure, function and potential for chemotherapy. *Biology of the Cell* **97**, 373–383.
- Bellofatto, V., Fairlamb, A.H., Henderson, G.B. and Cross, G.A.M. (1987). Biochemical changes associated with alpha-difluoromethylornithine uptake and resistance in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **25**, 227–238.
- Bendesky, A., Menendez, D. and Ostrosky-Wegman, P. (2002). Is metronidazole carcinogenic? *Mutation Research -Reviews in Mutation Research* **511**, 133–144.
- Berger, B.J., Carter, N.S. and Fairlamb, A.H. (1993). Polyamine and pentamidine metabolism in African trypanosomes. *Acta Tropica* **54**, 215–224.
- Berger, B.J., Carter, N.S. and Fairlamb, A.H. (1995). Characterization of pentamidine-resistant *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **69**, 289–298.
- Berger, B.J., Dai, W.W., Wang, H., Stark, R.E. and Cerami, A. (1996). Aromatic amino acid transamination and methionine recycling in trypanosomatids. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 4126–4130.
- Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D.C., Lennard, N.J., Caler, E., Hamlin, N.E., Haas, B., Bohme, W., Hannick, L., Aslett, M.A., Shallom, J., Marcello, L., Hou, L.H., Wickstead, B., Alsmark, U.C.M., Arrowsmith, C., Atkin, R.J., Barron, A.J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T.J., Churcher, C., Clark, L.N., Corton, C.H., Cronin, A., Davies, R.M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M.C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B.R., Hauser, H., Hostetter, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A.X., Koo, H., Larke, N., Landfear, S., Larkin, C., Leech, V., Line, A., Lord, A., MacLeod, A., Mooney, P.J., Moule, S., Martin, D.M.A., Morgan, G.W., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peacock, C.S., Peterson, J., Quail, M.A., Rabinowitsch, E., Rajandream, M.A., Reitter, C., Salzberg, S.L.,

- Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A.J., Talton, L., Turner, C.M.R., Tait, A., Tivey, A.R., Van Aken, S., Walker, D., Wanless, D., Wang, S.L., White, B., White, O., Whitehead, S., Woodward, J., Wortman, J., Adams, M.D., Embley, T.M., Gull, K., Ullu, E., Barry, J.D., Fairlamb, A.H., Oppendoes, F., Barret, B.G., Donelson, J.E., Hall, N., Fraser, C.M., Melville, S.E. and El Sayed, N.M. (2005). The genome of the African trypanosome *Trypanosoma brucei*. *Science* **309**, 416–422.
- Bitonti, A.J., Mccann, P.P. and Sjoerdsma, A. (1986). Necessity of antibody-response in the treatment of African trypanosomiasis with alpha-difluoromethylornithine. *Biochemical Pharmacology* **35**, 331–334.
- Bitter, W., Gerrits, H., Kieft, R. and Borst, P. (1998). The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. *Nature* **391**, 499–502.
- Bouvier, L.A., Silber, A.M., Lopes, C.G., Canepa, G.E., Miranda, M.R., Tonelli, R.R., Colli, W., Alves, M.J.M. and Pereira, C.A. (2004). Post genomic analysis of permeases from the amino acid auxin family in protozoan parasites. *Biochemical and Biophysical Research Communications* **321**, 547–556.
- Bowes, A.E., Samad, A.H., Jiang, P., Weaver, B. and Mellors, A. (1993). The acquisition of lysophosphatidylcholine by African trypanosomes. *Journal of Biological Chemistry* **268**, 13885–13892.
- Bray, P.G., Barrett, M.P., Ward, S.A. and de Koning, H.P. (2003). Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. *Trends in Parasitology* **19**, 232–239.
- Bringaud, F. and Baltz, T. (1992). A potential hexose transporter gene expressed predominantly in the blood-stream form of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **52**, 111–122.
- Bringaud, F. and Baltz, T. (1993). Differential regulation of 2 distinct families of glucose transporter genes in *Trypanosoma brucei*. *Molecular and Cellular Biology* **13**, 1146–1154.
- Bringaud, F. and Baltz, T. (1994). African trypanosome glucose-transporter genes – organization and evolution of a multigene family. *Molecular Biology and Evolution* **11**, 220–230.
- Brun, R., Schumacher, R., Schmid, C., Kunz, C. and Burri, C. (2001). The phenomenon of treatment failures in Human African Trypanosomiasis. *Tropical Medicine & International Health* **6**, 906–914.
- Burchmore, R.J.S., Wallace, L.J.M., Candlish, D., Al Salabi, M.I., Beal, P.R., Barrett, M.P., Baldwin, S.A. and de Koning, H.P. (2003). Cloning, heterologous expression, and *in situ* characterization of the first high affinity nucleobase transporter from a protozoan. *Journal of Biological Chemistry* **278**, 23502–23507.
- Burri, C. and Brun, R. (2003). Eflornithine for the treatment of human African trypanosomiasis. *Parasitology Research* **90**, S49–S52.

- Burri, C., Baltz, T., Giroud, C., Doua, F., Welker, H.A. and Brun, R. (1993). Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* **39**, 225–234.
- Burri, C., Stich, A. and Brun, R. (2004). Current chemotherapy of human African *Trypanosomiasis*. In: *The Trypanosomiasis* (I. Maudlin, P.H. Holmes and M.A. Miles, eds), pp. 403–420. Oxford: CABI.
- Byers, T.L., Casara, P. and Bitonti, A.J. (1992). Uptake of the anti-trypanosomal drug 5'-[(Z)-4-amino-2-butenyl]methylamino)-5'-deoxy-adenosine (Mdl 73811) by the purine transport-system of *Trypanosoma brucei brucei*. *Biochemical Journal* **283**, 755–758.
- Carter, N.S. and Fairlamb, A.H. (1993). Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* **361**, 173–176.
- Carter, N.S., Berger, B.J. and Fairlamb, A.H. (1995). Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. *Journal of Biological Chemistry* **270**, 28153–28157.
- Carter, N.S., Landfear, S.M. and Ullman, B. (2001). Nucleoside transporters of parasitic protozoa. *Trends in Parasitology* **17**, 142–145.
- Chowdhury, S.F., Villamor, V.B., Guerrero, R.H., Leal, I., Brun, R., Croft, S.L., Goodman, J.M., Maes, L., Ruiz-Perez, L.M., Pacanowska, D.G. and Gilbert, I.H. (1999). Design, synthesis, and evaluation of inhibitors of trypanosomal and leishmanial dihydrofolate reductase. *Journal of Medicinal Chemistry* **42**, 4300–4312.
- Chowdhury, S.F., Di Lucrezia, R., Guerrero, R.H., Brun, R., Goodman, J., Ruiz-Perez, L.M., Pacanowska, D.G. and Gilbert, I.H. (2001). Novel inhibitors of leishmanial dihydrofolate reductase. *Bioorganic & Medicinal Chemistry Letters* **11**, 977–980.
- Conroy, K., Eienthal, R., Game, S. and Holman, G. (1987). Sugar-transport in *Trypanosoma brucei*. *Biochemical Society Transactions* **15**, 1073.
- Coppens, I. and Courtoy, P.J. (2000). The adaptative mechanisms of *Trypanosoma brucei* for sterol homeostasis in its different life-cycle environments. *Annual Review of Microbiology* **54**, 129–156.
- Croft, S.L. (1999). Pharmacological approaches to antitrypanosomal chemotherapy. *Memorias do Instituto Oswaldo Cruz* **94**, 215–220.
- Croft, S.L., Seifert, K. and Duchene, M. (2003). Antiprotozoal activities of phospholipid analogues. *Molecular and Biochemical Parasitology* **126**, 165–172.
- Cunningham, M.L. and Beverley, S.M. (2001). Pteridine salvage throughout the *Leishmania* infectious cycle: implications for antifolate chemotherapy. *Molecular and Biochemical Parasitology* **113**, 199–213.
- Damper, D. and Patton, C.L. (1976a). Pentamidine transport and sensitivity in brucei-group trypanosomes. *Journal of Protozoology* **23**, 349–356.
- Damper, D. and Patton, C.L. (1976b). Pentamidine transport in *Trypanosoma brucei*—kinetics and specificity. *Biochemical Pharmacology* **25**, 271–276.

- Davis, R.H., Lieu, P. and Ristow, J.L. (1994). *Neurospora* mutants affecting polyamine-dependent processes and basic-amino-acid transport mutants resistant to the polyamine inhibitor, alpha-difluoromethylornithine. *Genetics* **138**, 649–655.
- Degreef, C. and Hamers, R. (1994). The serum resistance-associated (*sra*) gene of *Trypanosoma brucei rhodesiense* encodes a variant surface glycoprotein-like protein. *Molecular and Biochemical Parasitology* **68**, 277–284.
- Degreef, C., Chimfwembe, E., Wabacha, J.K., Songa, E.B. and Hamers, R. (1992). Only the serum-resistant blood-stream forms of *Trypanosoma brucei rhodesiense* express the serum resistance associated (SRA) protein. *Annales de la Société Belge de Médecine Tropicale* **72**, 13–21.
- de Koning, H.P. (2001a). Transporters in African trypanosomes: role in drug action and resistance. *International Journal for Parasitology* **31**, 512–522.
- de Koning, H.P. (2001b). Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. *Molecular Pharmacology* **59**, 586–592.
- de Koning, H.P. and Jarvis, S.M. (1998). A highly selective, high-affinity transporter for uracil in *Trypanosoma brucei brucei*: evidence for proton-dependent transport. *Biochemistry and Cell Biology (Biochimie et Biologie Cellulaire)* **76**, 853–858.
- de Koning, H.P. and Jarvis, S.M. (1999). Adenosine transporters in blood-stream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. *Molecular Pharmacology* **56**, 1162–1170.
- de Koning, H.P. and Jarvis, S.M. (2001). Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. *Acta Tropica* **80**, 245–250.
- de Koning, H.P., Watson, C.J. and Jarvis, S.M. (1998). Characterization of a nucleoside proton symporter in procyclic *Trypanosoma brucei brucei*. *Journal of Biological Chemistry* **273**, 9486–9494.
- de Koning, H.P., Anderson, L.F., Stewart, M.L., Burchmore, R.J.S., Wallace, L.J.M. and Barrett, M.P. (2004). The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in African trypanosomes. *Antimicrobial Agents and Chemotherapy* **48**, 1515–1519.
- de Koning, H.P., Bridges, D.J. and Burchmore, R.J. (2005). Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. *FEMS Microbiology Reviews* **29**, 987–1020.
- Denise, H. and Barrett, M.P. (2001). Uptake and mode of action of drugs used against sleeping sickness. *Biochemical Pharmacology* **61**, 1–5.
- Docampo, R. and Moreno, S.N.J. (1986). Free-radical metabolism of anti-parasitic agents. *Federation Proceedings* **45**, 2471–2476.
- Doua, F., Miezán, T.W., Singaro, J.R.S., Yapó, F.B. and Baltz, T. (1996). The efficacy of pentamidine in the treatment of early late stage *Trypanosoma*

- brucei gambiense* trypanosomiasis. *American Journal of Tropical Medicine and Hygiene* **55**, 586–588.
- Drews, J. (2004). Paul Ehrlich: magister Mundi. *Nature Reviews Drug Discovery* **3**, 797–801.
- Eisenthal, R. and Cornish-Bowden, A. (1998). Prospects for antiparasitic drugs The case of *Trypanosoma brucei*, the causative agent of African sleeping sickness. *Journal of Biological Chemistry* **273**, 5500–5505.
- Eisenthal, R., Game, S. and Holman, G.D. (1989). Specificity and kinetics of hexose-transport in *Trypanosoma brucei*. *Biochimica et Biophysica Acta* **985**, 81–89.
- El Fadili, A., Kundig, C., Roy, G. and Ouellette, M. (2004). Inactivation of the *Leishmania tarentolae* pterin transporter (*BT1*) and reductase (*PTR1*) genes leads to viable parasites with changes in folate metabolism and hypersensitivity to the antifolate methotrexate. *Journal of Biological Chemistry* **279**, 18575–18582.
- El Rayah, I.E., Kaminsky, R., Schmid, C. and El Malik, K.H. (1999). Drug resistance in Sudanese *Trypanosoma evansi*. *Veterinary Parasitology* **80**, 281–287.
- Enanga, B., Keita, M., Chauviere, G., Dumas, M. and Bouteille, B. (1998). Megazol combined with suramin: a chemotherapy regimen which reversed the CNS pathology in a model of human African trypanosomiasis in mice. *Tropical Medicine & International Health* **3**, 736–741.
- Enanga, B., Ariyanayagam, M.R., Stewart, M.L. and Barrett, M.P. (2003). Activity of megazol, a trypanocidal nitroimidazole, is associated with DNA damage. *Antimicrobial Agents and Chemotherapy* **47**, 3368–3370.
- Engstler, M., Thilo, L., Weise, F., Grunfelder, C.G., Schwarz, H., Boshart, M. and Overath, P. (2004). Kinetics of endocytosis and recycling of the GPI-anchored variant surface glycoprotein in *Trypanosoma brucei*. *Journal of Cell Science* **117**, 1105–1115.
- Erwin, B.G. and Pegg, A.E. (1982). Uptake of alpha-difluoromethylornithine by mouse fibroblasts. *Biochemical Pharmacology* **31**, 2820–2823.
- Fairlamb, A.H. (2003). Chemotherapy of human African trypanosomiasis: current and future prospects. *Trends in Parasitology* **19**, 488–494.
- Fairlamb, A.H. and Bowman, I.B.R. (1977). *Trypanosoma brucei*—suramin and other trypanocidal compounds effects on Sn-glycerol-3-phosphate oxidase. *Experimental Parasitology* **43**, 353–361.
- Fairlamb, A.H. and Bowman, I.B.R. (1980). Uptake of the trypanocidal drug suramin by blood-stream forms of *Trypanosoma brucei* and its effect on respiration and growth-rate *in vivo*. *Molecular and Biochemical Parasitology* **1**, 315–333.
- Fairlamb, A.H. and Le Quesne, S.A. (1997). Polyamine metabolism in trypanosomes. In: *Trypanosomiasis and Leishmaniasis* (G. Hide *et al.*, eds), pp. 149–162. Wallingford: CAB International.

- Fairlamb, A.H., Oppendoes, F.R. and Borst, P. (1977). New approach to screening drugs for activity against African trypanosomes. *Nature* **265**, 270–271.
- Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985). Trypanothione – a novel bis(glutathionyl)spermidine cofactor for glutathione-reductase in trypanosomatids. *Science* **227**, 1485–1487.
- Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987). *In vivo* effects of difluoromethylornithine on trypanothione and polyamine levels in blood-stream forms of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **24**, 185–191.
- Fairlamb, A.H., Henderson, G.B. and Cerami, A. (1989). Trypanothione is the primary target for arsenical drugs against African trypanosomes. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 2607–2611.
- Fairlamb, A.H., Carter, N.S., Cunningham, M. and Smith, K. (1992a). Characterization of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology* **53**, 213–222.
- Fairlamb, A.H., Smith, K. and Hunter, K.J. (1992b). The interaction of arsenical drugs with dihydrolipoamide and dihydrolipoamide dehydrogenase from arsenical resistant and sensitive strains of *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **53**, 223–231.
- Fevre, E.M., Coleman, P.G., Welburn, S.C. and Maudlin, I. (2004). Re-analyzing the 1900–1920 sleeping sickness epidemic in Uganda. *Emerging Infectious Diseases* **10**, 567–573.
- Filardi, L.S. and Brener, Z. (1987). Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 755–759.
- Frommel, T.O. and Balber, A.E. (1987). Flow cytofluorometric analysis of drug accumulation by multidrug-resistant *Trypanosoma brucei brucei* and *T. b. rhodesiense*. *Molecular and Biochemical Parasitology*, **26**, 183–191.
- Fry, A.J., Towner, P., Holman, G.D. and Eienthal, R. (1993). Transport of D-fructose and its analogs by *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **60**, 9–18.
- Geiser, F., Luscher, A., de Koning, H.P., Seebeck, T. and Maser, P. (2005). Molecular pharmacology of adenosine transport in *Trypanosoma brucei*: P1/P2 revisited. *Molecular Pharmacology* **68**, 589–595.
- Gerrits, H., Musmann, R., Bitter, W., Kieft, R. and Borst, P. (2002). The physiological significance of transferrin receptor variations in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **119**, 237–247.
- Ghoda, L., Phillips, M.A., Bass, K.E., Wang, C.C. and Coffino, P. (1990). Trypanosome ornithine decarboxylase is stable because it lacks sequences found in the carboxyl terminus of the mouse enzyme which target the

- latter for intracellular degradation. *Journal of Biological Chemistry* **265**, 11823–11826.
- Gibaud, S., Gaia, A. and Astier, A. (2002). Slow-release melarsoprol micro-particles. *International Journal of Pharmaceutics* **243**, 161–166.
- Gottesdiener, K.M. (1994). A new VSG expression site-associated gene (*ESAG*) in the promoter region of *Trypanosoma brucei* encodes a protein with 10 potential transmembrane domains. *Molecular and Biochemical Parasitology* **63**, 143–151.
- Gourbal, B., Sonuc, N., Bhattacharjee, H., Legare, D., Sundar, S., Ouellette, M., Rosen, B.P. and Mukhopadhyay, R. (2004). Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. *Journal of Biological Chemistry* **279**, 31010–31017.
- Grant, P.T. and Fulton, J.D. (1956). Glucose catabolism by strains of *Trypanosoma rhodesiense*. *Biochemical Journal* **63**, 8–9.
- Green, H.P., Portela, M.D.M., St Jean, E.N., Lugli, E.B. and Raper, J. (2003). Evidence for a *Trypanosoma brucei* lipoprotein scavenger receptor. *Journal of Biological Chemistry* **278**, 422–427.
- Hall, J.E. and Seed, J.R. (1981). Quantitation of aromatic amino-acid catabolites in urine of mice acutely infected with *Trypanosoma brucei gambiense*. *Comparative Biochemistry and Physiology B—Biochemistry & Molecular Biology* **69**, 791–796.
- Hall, J.E. and Seed, J.R. (1984). Increased urinary-excretion of aromatic amino-acid catabolites by *Microtus montanus* chronically infected with *Trypanosoma brucei gambiense*. *Comparative Biochemistry and Physiology B—Biochemistry & Molecular Biology* **77**, 755–760.
- Hansen, B.D. (1979). *Trypanosoma gambiense*—Membrane-transport of amino-acids. *Experimental Parasitology* **48**, 296–304.
- Hardy, L.W., Matthews, W., Nare, B. and Beverley, S.M. (1997). Biochemical and genetic tests for inhibitors of *Leishmania* pteridine pathways. *Experimental Parasitology* **87**, 157–169.
- Hasne, M.P. and Barrett, M.P. (2000a). Drug uptake via nutrient transporters in *Trypanosoma brucei*. *Journal of Applied Microbiology* **89**, 697–701.
- Hasne, M.P. and Barrett, M.P. (2000b). Transport of methionine in *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **111**, 299–307.
- Hasne, M.P. and Ullman, B. (2005). Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. *Journal of Biological Chemistry* **280**, 15188–15194.
- Hawking, F.J. and Smiles, S.J. (1941). The distribution of 4:4'-diamidino-stilbene in trypanosomes and mice as shown by fluorescence. *Annals of Tropical Medicine and Parasitology* **35**, 45–50.
- Henderson, G.B., Ulrich, P., Fairlamb, A.H., Rosenberg, I., Pereira, M., Sela, M. and Cerami, A. (1988). Subversive substrates for the enzyme trypanothione disulfide reductase—alternative approach to chemotherapy

- of Chagas-disease. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 5374–5378.
- Henriques, C., Sanchez, M.A., Tryon, R. and Landfear, S.M. (2003). Molecular and functional characterization of the first nucleobase transporter gene from African trypanosomes. *Molecular and Biochemical Parasitology* **130**, 101–110.
- Hofer, A., Steverding, D., Chabes, A., Brun, R. and Thelander, L. (2001). *Trypanosoma brucei* CTP synthetase: a target for the treatment of African sleeping sickness. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 6412–6416.
- Huynh, T.T., Huynh, V.T., Harmon, M.A. and Phillips, M.A. (2003). Gene knockdown of gamma-glutamylcysteine synthetase by RNAi in the parasitic protozoon *Trypanosoma brucei* demonstrates that it is an essential enzyme. *Journal of Biological Chemistry* **278**, 39794–39800.
- Hyde, J.E. (2002). Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes and Infection* **4**, 165–174.
- Iten, M., Matovu, E., Brun, R. and Kaminsky, R. (1995). Innate lack of susceptibility of Ugandan *Trypanosoma brucei rhodesiense* to DL-alpha-difluoromethylornithine (DFMO). *Tropical Medicine and Parasitology* **46**, 190–194.
- Jaffe, J.J. (1963). *In vivo* activity of L-azaserine against *Trypanosoma equiperdum*. *Journal of Protozoology* **10**, 431–436.
- Jaffe, J.J., Voorheis, H.P. and McCormac, J.J. (1969). Comparative effects of diverse mammalian sera on uptake and protein incorporation of ¹⁴C-labelled amino acids by blood stream forms of *T. brucei* and *T. rhodesiense* *in vitro*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **63**, 118–125.
- James, D.M. and Born, G.V.R. (1980). Uptake of purine-bases and nucleosides in African trypanosomes. *Parasitology* **81**, 383–393.
- Janssens, P.G. and Demuynck, A. (1977). Clinical trials with nifurtimox in African trypanosomiasis. *Annales de la Société Belge de Médecine Tropicale* **57**, 475–480.
- Jennings, F.W. (1991). Chemotherapy of CNS-trypanosomiasis – the combined use of the arsenicals and nitrocompounds. *Tropical Medicine and Parasitology* **42**, 139–142.
- Jennings, F.W., Atouguia, J.M. and Murray, M. (1993). Topical melarso-prol for trypanosomiasis. *Lancet* **341**, 1341–1342.
- Kaminsky, R., Iten, M., Mett, H., Littlewood, A. and Brun, R. (1996). Alterations in ornithine decarboxylase characteristics account for tolerance of *Trypanosoma brucei rhodesiense* to DL-alpha-difluoromethylornithine (DFMO). *Tropical Medicine & International Health* **1**, A32–A33.
- Keiser, J., Stich, A. and Burri, C. (2001). New drugs for the treatment of human African trypanosomiasis: research and development. *Trends in Parasitology* **17**, 42–49.

- Khabnadideh, S., Pez, D., Musso, A., Brun, R., Perez, L.M.R., Gonzalez-Pacanowska, D. and Gilbert, I.H. (2005). Design, synthesis and evaluation of 2,4-diaminoquinazolines as inhibitors of trypanosomal and leishmanial dihydrofolate reductase. *Bioorganic & Medicinal Chemistry* **13**, 2637–2649.
- Kinabo, L.D.B. (1993). Pharmacology of existing drugs for animal trypanosomiasis. *Acta Tropica* **54**, 169–183.
- Klenke, B., Stewart, M.L., Barrett, M.P., Brun, R. and Gilbert, I.H. (2001). Synthesis and biological evaluation of *s*-triazine substituted polyamines as potential new anti-trypanosomal drugs. *Journal of Medicinal Chemistry* **44**, 3440–3452.
- Kralova, I., Rigden, D.J., Oppendoes, F.R. and Michels, P.A.M. (2000). Glycerol kinase of *Trypanosoma brucei*—Cloning, molecular characterization and mutagenesis. *European Journal of Biochemistry* **267**, 2323–2333.
- Kundig, C., Haimeur, A., Legare, D., Papadopolou, B. and Ouellette, M. (1999). Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*. *EMBO Journal* **18**, 2342–2351.
- Lamour, N., Riviere, L., Coustou, V., Coombs, G.H., Barrett, M.P. and Bringaud, F. (2005). Proline metabolism in procyclic *Trypanosoma brucei* is down-regulated in the presence of glucose. *Journal of Biological Chemistry* **280**, 11902–11910.
- Landfear, S.M. (2001). Molecular genetics of nucleoside transporters in *Leishmania* and African trypanosomes. *Biochemical Pharmacology* **62**, 149–155.
- Landfear, S.M., Ullman, B., Carter, N.S. and Sanchez, M.A. (2004). Nucleoside and nucleobase transporters in parasitic protozoa. *Eukaryotic Cell* **3**, 245–254.
- Leandro, C. and Campino, L. (2003). Leishmaniasis: efflux pumps and chemoresistance. *International Journal of Antimicrobial Agents* **22**, 352–357.
- Legare, D., Cayer, S., Singh, A.K., Richard, D., Papadopolou, B. and Ouellette, M. (2001). ABC proteins of *Leishmania*. *Journal of Bioenergetics and Biomembranes* **33**, 469–474.
- Legros, D., Evans, S., Maiso, F., Enyaru, J.C.K. and Mbulamberi, D. (1999). Risk factors for treatment failure after melarsoprol for *Trypanosoma brucei gambiense* trypanosomiasis in Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**, 439–442.
- Legros, D., Ollivier, G., Gastellu-Etchegorry, M., Paquet, C., Burri, C., Jannin, J. and Buscher, P. (2002). Treatment of human African trypanosomiasis—present situation and needs for research and development. *Lancet Infectious Diseases* **2**, 437–440.
- Lemley, C., Yan, S.F., Dole, V.S., Madhubala, R., Cunningham, M.L., Beverley, S.M., Myler, P.J. and Stuart, K.D. (1999). The *Leishmania*

- donovani* LD1 locus gene *ORFG* encodes a biopterin transporter (BT1). *Molecular and Biochemical Parasitology* **104**, 93–105.
- LeQuesne, S.A. and Fairlamb, A.H. (1996). Regulation of a high-affinity diamine transport system in *Trypanosoma cruzi* epimastigotes. *Biochemical Journal* **316**, 481–486.
- L'hostis, C., Geindre, M. and Deshusses, J. (1993). Active-transport of L-proline in the protozoan parasite *Trypanosoma brucei brucei*. *Biochemical Journal* **291**, 297–301.
- Li, F., Hua, S.B., Wang, C.C. and Gottesdiener, K.M. (1998). *Trypanosoma brucei brucei*: characterization of an ODC null bloodstream form mutant and the action of alpha-difluoromethylornithine. *Experimental Parasitology* **88**, 255–259.
- Liu, Z.J., Carlbrey, J.M., Agre, P. and Rosen, B.P. (2004). Arsenic trioxide uptake by human and rat aquaglyceroporins. *Biochemical and Biophysical Research Communications* **316**, 1178–1185.
- Loiseau, P.M., Czok, M., Chauffert, O., Bourass, J. and Letourneux, Y. (1998). Studies on lipidomimetic derivatives of alpha-difluoromethylornithine (DFMO) to enhance the bioavailability in a *Trypanosoma brucei* murine trypanosomiasis model. *Parasite – Journal de la Société Française de Parasitologie* **5**, 239–246.
- Maser, P. and Kaminsky, R. (1998). Identification of three ABC transporter genes in *Trypanosoma brucei* spp. *Parasitology Research* **84**, 106–111.
- Maser, P., Sutterlin, C., Kralli, A. and Kaminsky, R. (1999). A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **285**, 242–244.
- Maser, P., Vogel, D., Schmid, C., Raz, B. and Kaminsky, R. (2001). Identification and characterization of trypanocides by functional expression of an adenosine transporter from *Trypanosoma brucei* in yeast. *Journal of Molecular Medicine* **79**, 121–127.
- Maser, P., Luscher, A. and Kaminsky, R. (2003). Drug transport and drug resistance in African trypanosomes. *Drug Resistance Updates* **6**, 281–290.
- Matovu, E., Enyaru, J.C.K., Legros, D., Schmid, C., Seebeck, T. and Kaminsky, R. (2001). Melarsoprol refractory *T. b. gambiense* from Omugo, north-western Uganda. *Tropical Medicine & International Health* **6**, 407–411.
- Matovu, E., Stewart, M.L., Geiser, F., Brun, R., Maser, P., Wallace, L.J.M., Burchmore, R.J., Enyaru, J.C.K., Barrett, M.P., Kaminsky, R., Seebeck, T. and de Koning, H.P. (2003). Mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. *Eukaryotic Cell* **2**, 1003–1008.
- McCulloch, R. (2004). Antigenic variation in African trypanosomes: monitoring progress. *Trends in Parasitology* **20**, 117–121.
- Mellors, A. and Samad, A. (1989). The acquisition of lipids by African trypanosomes. *Parasitology Today* **5**, 239–244.

- Meshnick, S.R., Blobstein, S.H., Grady, R.W. and Cerami, A. (1978). Approach to development of new drugs for African trypanosomiasis. *Journal of Experimental Medicine* **148**, 569–579.
- Millet, R., Maes, L., Landry, V., Sergheraert, C. and Davioud-Charvet, E. (2002). Antitrypanosomal activities and cytotoxicity of 5-nitro-2-furancarbohydrazides. *Bioorganic & Medicinal Chemistry Letters* **12**, 3601–3604.
- Moens, F., Dewilde, M. and Ngato, K. (1984). Clinical trial of nifurtimox in human African trypanosomiasis. *Annales de la Société Belge de Médecine Tropicale* **64**, 37–43.
- Moore, A. and Richer, M. (2001). Re-emergence of epidemic sleeping sickness in southern Sudan. *Tropical Medicine & International Health* **6**, 342–347.
- Murta, S.M.F., Gazzinelli, R.T., Brener, Z. and Romanha, A.J. (1998). Molecular characterization of susceptible and naturally resistant strains of *Trypanosoma cruzi* to benznidazole and nifurtimox. *Molecular and Biochemical Parasitology* **93**, 203–214.
- Mussmann, R., Janssen, H., Calafat, J., Engstler, M., Ansorge, I., Clayton, C. and Borst, P. (2003). The expression level determines the surface distribution of the transferrin receptor in *Trypanosoma brucei*. *Molecular Microbiology* **47**, 23–35.
- Mussmann, R., Engstler, M., Gerrits, H., Kieft, R., Toaldo, C.B., Onderwater, J., Koerten, H., van Luenen, H.G.A.M. and Borst, P. (2004). Factors affecting the level and localization of the transferrin receptor in *Trypanosoma brucei*. *Journal of Biological Chemistry* **279**, 40690–40698.
- Mutomba, M.C. and Wang, C.C. (1996). Effects of aphidicolin and hydroxyurea on the cell cycle and differentiation of *Trypanosoma brucei* blood-stream forms. *Molecular and Biochemical Parasitology* **80**, 89–102.
- Mutugi, M.W., Boid, R. and Luckins, A.G. (1994). Experimental induction of suramin-resistance in cloned and uncloned stocks of *Trypanosoma evansi* using immunosuppressed and immunocompetent mice. *Tropical Medicine and Parasitology* **45**, 232–236.
- Mutugi, M.W., Boid, R. and Luckins, A.G. (1995). Differences in cloning and sub-cloning success rates in 4 stocks of *Trypanosoma evansi* and variation in suramin resistance of the clones. *Veterinary Parasitology* **60**, 213–220.
- Natto, M.J., Wallace, L.J.M., Candlish, D., Al Salabi, M.I., Coutts, S.E. and de Koning, H.P. (2005). *Trypanosoma brucei*: expression of multiple purine transporters prevents the development of allopurinol resistance. *Experimental Parasitology* **109**, 80–86.
- Nok, A.J. and Nock, I.H. (2002). Transferrin coupled azanthraquinone enhances the killing effect on trypanosomes. The role of lysosomal mannosidase. *Parasite- Journal de la Société Française de Parasitologie* **9**, 375–379.

- Nolan, D.P., Rolin, S., Rodriguez, J.R., Van den Abbeele, J. and Pays, E. (2000). Slender and stumpy bloodstream forms of *Trypanosoma brucei* display a differential response to extracellular acidic and proteolytic stress. *European Journal of Biochemistry* **267**, 18–27.
- Olbrich, C., Gessner, A., Schroder, W., Kayser, O. and Muller, R.H. (2004). Lipid drug conjugate nanoparticles of the hydrophilic drug dimina-zene—cytotoxicity testing and mouse serum adsorption. *Journal of Controlled Release* **96**, 425–435.
- Oppendoes, F.R. (1987). Compartmentation of carbohydrate metabolism in trypanosomes. *Annual Review of Microbiology* **41**, 127–151.
- Oppendoes, F.R. and Borst, P. (1977). Localization of 9 glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*—glycosome. *FEBS Letters* **80**, 360–364.
- Oppendoes, F.R. and Fairlamb, A.H. (1977). Trypanocidal action of sali-cylhydroxamic acid plus glycerol. *Tropical and Geographical Medicine* **29**, 450–451.
- Oppendoes, F.R., Borst, P. and Fonck, K. (1976). Potential use of inhibitors of glycerol-3-phosphate oxidase for chemotherapy of African trypanosomiasis. *FEBS Letters* **62**, 169–172.
- Oppendoes, F.R., Borst, P. and Spits, H. (1977). Particle-bound enzymes in blood-stream form of *Trypanosoma brucei*. *European Journal of Biochemistry* **76**, 21–28.
- Ouellette, M., Legare, D. and Papadopolou, B. (2001). Multidrug resistance and ABC transporters in parasitic protozoa. *Journal of Molecular Microbiology and Biotechnology* **3**, 201–206.
- Ouellette, M., Drummelsmith, J., El Fadili, A., Kundig, C., Richard, D. and Roy, G. (2002). Pterin transport and metabolism in *Leishmania* and related trypanosomatid parasites. *International Journal for Parasitology* **32**, 385–398.
- Owen, M.J. and Voorheis, H.P. (1976). Active-site-directed inhibition of plasma-membrane carrier transporting short-chain, neutral amino-acids into *Trypanosoma brucei*. *European Journal of Biochemistry* **62**, 619–624.
- Owolabi, O.A., Wilson, C., Molyneux, D.H. and Pentreath, V.W. (1990). Trypanocidal effects of catecholamines and indolealkylamines. *Annals of Tropical Medicine and Parasitology* **84**, 127–131.
- Pal, A., Hall, B.S. and Field, M.C. (2002). Evidence for a non-LDL-mediated entry route for the trypanocidal drug suramin in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **122**, 217–221.
- Papadopolou, B., Roy, G., Breton, M., Kundig, C., Dumas, C., Fillion, I., Singh, A.K., Olivier, M. and Ouellette, M. (2002). Reduced infectivity of a *Leishmania donovani* bipterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infection and Immunity* **70**, 62–68.
- Pépin, J. and Milord, F. (1994). The treatment of human African trypanosomiasis. *Advances in Parasitology* **33**, 1–47.

- Pépin, J., Milord, F., Mpia, B., Meurice, F., Ethier, L., Degroof, D. and Bruneel, H. (1989). An open clinical trial of nifurtimox for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness in central Zaire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83**, 514–517.
- Peregrine, A.S. and Mamman, M. (1993). Pharmacology of diminazene—a review. *Acta Tropica* **54**, 185–203.
- Perez-Morga, D., Vanhollebeke, B., Paturiaux-Hanocq, F., Nolan, D.P., Lins, L., Homble, F., Vanhamme, L., Tebabi, P., Pays, A., Poelvoorde, P., Jacquet, A., Brasseur, R. and Pays, E. (2005). Apolipoprotein L-1 promotes trypanosome lysis by forming pores in lysosomal membranes. *Science* **309**, 469–472.
- Perez-Victoria, J.M., Perez-Victoria, F.J., Parodi-Talice, A., Jimenez, I.A., Ravelo, A.G., Castanys, S. and Gamarro, F. (2001). Alkyl-lysophospholipid resistance in multi drug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. *Antimicrobial Agents and Chemotherapy* **45**, 2468–2474.
- Perez-Victoria, F.J., Castanys, S. and Gamarro, F. (2003a). *Leishmania donovani* resistance to miltefosine involves a defective inward translocation of the drug. *Antimicrobial Agents and Chemotherapy* **47**, 2397–2403.
- Perez-Victoria, F.J., Gamarro, F., Ouellette, M. and Castanys, S. (2003b). Functional cloning of the miltefosine transporter—a novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *Journal of Biological Chemistry* **278**, 49965–49971.
- Pez, D., Leal, I., Zuccotto, F., Boussard, C., Brun, R., Croft, S.L., Yardley, V., Perez, L.M.R., Pacanowska, D.G. and Gilbert, I.H. (2003). 2,4-Diaminopyrimidines as inhibitors of leishmanial and trypanosomal dihydrofolate reductase. *Bioorganic & Medicinal Chemistry* **11**, 4693–4711.
- Phelouzat, M.A., Basselin, M., Lawrence, F. and Robert-Gero, M. (1995). Sinefungin shares adomet-uptake system to enter *Leishmania donovani* promastigotes. *Biochemical Journal* **305**, 133–137.
- Phillips, M.A. and Wang, C.C. (1987). A *Trypanosoma brucei* mutant resistant to alpha-difluoromethylornithine. *Molecular and Biochemical Parasitology* **22**, 9–17.
- Raz, B., Iten, M., Grether Buhler, Y., Kaminsky, R. and Brun, R. (1997). The Alamar Blue[®] assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Tropica* **68**, 139–147.
- Richard, D., Kundig, C. and Ouellette, M. (2002). A new type of high affinity folic acid transporter in the protozoan parasite *Leishmania* and deletion of its gene in methotrexate-resistant cells. *Journal of Biological Chemistry* **277**, 29460–29467.
- Richard, D., Leprohon, P., Drummelsmith, J. and Ouellette, M. (2004). Growth phase regulation of the main folate transporter of *Leishmania*

- infantum* and its role in methotrexate resistance. *Journal of Biological Chemistry* **279**, 54494–54501.
- Ross, C.A. and Barns, A.M. (1996). Alteration to one of three adenosine transporters is associated with resistance to cymelarsan in *Trypanosoma evansi*. *Parasitology Research* **82**, 183–188.
- Samad, A., Licht, B., Stalmach, M.E. and Mellors, A. (1988). Metabolism of phospholipids and lysophospholipids by *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **29**, 159–169.
- Sanchez, M.A., Ullman, B., Landfear, S.M. and Carter, N.S. (1999). Cloning and functional expression of a gene encoding a P1 type nucleoside transporter from *Trypanosoma brucei*. *Journal of Biological Chemistry* **274**, 30244–30249.
- Sanchez, M.A., Tryon, R., Green, J., Boor, J. and Landfear, S.M. (2002). Six related nucleoside nucleobase transporters from *Trypanosoma brucei* exhibit distinct biochemical functions. *Journal of Biological Chemistry* **277**, 21499–21504.
- Sanchez, M.A., Drutman, S., van Ampting, M., Matthews, K. and Landfear, S.M. (2004). A novel purine nucleoside transporter whose expression is up-regulated in the short stumpy form of the *Trypanosoma brucei* life cycle. *Molecular and Biochemical Parasitology* **136**, 265–272.
- Sands, M., Kron, M.A. and Brown, R.B. (1985). Pentamidine – a review. *Reviews of Infectious Diseases* **7**, 625–634.
- Schell, D., Borowy, N.K. and Overath, P. (1991a). Transferrin is a growth-factor for the blood-stream form of *Trypanosoma brucei*. *Parasitology Research* **77**, 558–560.
- Schell, D., Evers, R., Preis, D., Ziegelbauer, K., Kiefer, H., Lottspeich, F., Cornelissen, A.W.C.A. and Overath, P. (1991b). A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene-expression site. *EMBO Journal* **10**, 1061–1066.
- Schmidt, A., Clayton, C.E. and Krauth-Siegel, R.L. (2002). Silencing of the thioredoxin gene in *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **125**, 207–210.
- Schnauffer, A., Domingo, G.J. and Stuart, K. (2002). Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA. *International Journal for Parasitology* **32**, 1071–1084.
- Scott, A.G., Tait, A. and Turner, C.M.R. (1996). Characterisation of cloned lines of *Trypanosoma brucei* expressing stable resistance to MelCy and suramin. *Acta Tropica* **60**, 251–262.
- Scott, A.G., Tait, A. and Turner, C.M.R. (1997). *Trypanosoma brucei*: lack of cross-resistance to melarsoprol *in vitro* by cymelarsan-resistant parasites. *Experimental Parasitology* **86**, 181–190.
- Seifert, K., Matu, S., Perez-Victoria, F.J., Castanys, S., Gamarro, F. and Croft, S.L. (2003). Characterisation of *Leishmania donovani* promastigotes

- resistant to hexadecylphosphocholine (miltefosine). *International Journal of Antimicrobial Agents* **22**, 380–387.
- Seyfang, A. and Duszenko, M. (1991). Specificity of glucose transport in *Trypanosoma brucei* – effective inhibition by phloretin and cytochalasin-B. *European Journal of Biochemistry* **202**, 191–196.
- Seyfang, A. and Duszenko, M. (1993). Functional reconstitution of the *Trypanosoma brucei* plasma-membrane D-glucose transporter. *European Journal of Biochemistry* **214**, 593–597.
- Shahi, S.K., Krauth-Siegel, R.L. and Clayton, C.E. (2002). Overexpression of the putative thiol conjugate transporter TbMRPA causes melarsoprol resistance in *Trypanosoma brucei*. *Molecular Microbiology* **43**, 1129–1138.
- Shuaibu, M.N., Kanbara, H., Yanagi, T., Ichinose, A., Ameh, D.A., Bonire, J.J. and Nok, A.J. (2003). *In vitro* trypanocidal activity of dibutyltin dichloride and its fatty acid derivatives. *Parasitology Research* **91**, 5–11.
- Simpson, L. (1986). Kinetoplast DNA in trypanosomid flagellates. *International Review of Cytology—A Survey of Cell Biology* **99**, 119–179.
- Soulere, L., Hoffmann, P., Bringaud, F. and Perie, J. (1999). Uptake of NO-releasing drugs by the P2 nucleoside transporter in trypanosomes. *Brazilian Journal of Medical and Biological Research* **32**, 1447–1452.
- Soulere, L., Hoffmann, P., Bringaud, F. and Perie, J. (2000). Synthesis and uptake of nitric oxide-releasing drugs by the P2 nucleoside transporter in *Trypanosoma equiperdum*. *Bioorganic & Medicinal Chemistry Letters* **10**, 1347–1350.
- Stanghellini, A. and Josenando, T. (2001). The situation of sleeping sickness in Angola: a calamity. *Tropical Medicine & International Health* **6**, 330–334.
- Steinborn, K., Szallies, A., Mecke, D. and Duszenko, M. (2000). Cloning, heterologous expression and kinetic analysis of glycerol kinase (TbGLK1) from *Trypanosoma brucei*. *Biological Chemistry* **381**, 1071–1077.
- Steverding, D., Stierhof, Y.D., Chaudhri, M., Ligtenberg, M., Schell, D., Becksickinger, A.G. and Overath, P. (1994). ESAG-6 and ESAG-7 products of *Trypanosoma brucei* form a transferrin-binding protein complex. *European Journal of Cell Biology* **64**, 78–87.
- Steverding, D., Stierhof, Y.D., Fuchs, H., Tauber, R. and Overath, P. (1995). Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. *Journal of Cell Biology* **131**, 1173–1182.
- Stewart, M.L., Bueno, G.J., Baliani, A., Klenke, B., Brun, R., Brock, J.M., Gilbert, I.H. and Barrett, M.P. (2004). Trypanocidal activity of melamine-based nitroheterocycles. *Antimicrobial Agents and Chemotherapy* **48**, 1733–1738.
- Stewart, M.L., Krishna, S., Burchmore, R.J.S., Brun, R., de Koning, H.P., Boykin, D.W., Tidwell, R.R., Hall, J.E. and Barrett, M.P. (2005). Detection of arsenical drug resistance in *Trypanosoma brucei* with a simple fluorescence test. *Lancet* **366**, 486–487.

- Stoppani, A.O.M. (1999). The chemotherapy of Chagas disease. *Medicina Buenos Aires* **59**, 147–165.
- Sturk, L.M., Brock, J.L., Bagnell, C.R., Hall, J.E. and Tidwell, R.R. (2004). Distribution and quantitation of the anti-trypansomal diamidine 2, 5-bis(4-amidinophenyl)furan (DB75) and its *N*-methoxy prodrug DB289 in murine brain tissue. *Acta Tropica* **91**, 131–143.
- Sufrin, J.R., Rattendi, D., Spiess, A.J., Lane, S., Marasco, C.J. and Bacchi, C.J. (1996). Antitrypanosomal activity of purine nucleosides can be enhanced by their conversion to *O*-acetylated derivatives. *Antimicrobial Agents and Chemotherapy* **40**, 2567–2572.
- Susperregui, J., Petsom, A., Bayle, M., Lain, G., Giroud, C., Baltz, T. and Deleris, G. (1997). The *in vitro* trypanocidal activity of organotin compounds. *European Journal of Medicinal Chemistry* **32**, 123–128.
- Susperregui, J., Bayle, M., Lain, G., Giroud, C., Baltz, T. and Deleris, G. (1999). Synthesis and evaluation of the *in vivo* trypanocidal activity of water soluble organotin compounds. *European Journal of Medicinal Chemistry* **34**, 617–623.
- Terkule, B.H. and Oppenheims, F.R. (1991). Glucose-uptake by *Trypanosoma brucei* – rate-limiting steps in glycolysis and regulation of the glycolytic flux. *Journal of Biological Chemistry* **266**, 857–862.
- Tetaud, E., Barrett, M.P., Bringaud, F. and Baltz, T. (1997). Kinetoplastid glucose transporters. *Biochemical Journal* **325**, 569–580.
- Thissen, J.A. and Wang, C.C. (1991). Maintenance of internal pH and an electrochemical gradient in *Trypanosoma brucei*. *Experimental Parasitology* **72**, 243–251.
- Tsuhako, M.H., Alves, M.J.M., Colli, W., Filardi, L.S., Brener, Z. and Augusto, O. (1991). Comparative studies of nifurtimox uptake and metabolism by drug-resistant and susceptible strains of *Trypanosoma cruzi*. *Comparative Biochemistry and Physiology C – Pharmacology Toxicology & Endocrinology* **99**, 317–321.
- Tye, C.K., Kasinathan, G., Barrett, M.P., Brun, R., Doyle, V.E., Fairlamb, A.H., Weaver, R. and Gilbert, I.H. (1998). An approach to use an unusual adenosine transporter to selectively deliver polyamine analogues to trypanosomes. *Bioorganic & Medicinal Chemistry Letters* **8**, 811–816.
- Uzcategui, N.L., Szallies, A., Pavlovic-Djuranovic, S., Palmada, M., Figarella, K., Boehmer, C., Lang, F., Beitz, E. and Duzenko, M. (2004). Cloning, heterologous expression, and characterization of three aquaglyceroporins from *Trypanosoma brucei*. *Journal of Biological Chemistry* **279**, 42669–42676.
- Vanbogaert, I., Haemers, A., Bollaert, W., Vanmeirvenne, N., Brun, R., Smith, K. and Fairlamb, A.H. (1993). Synthesis and antitrypanosomal evaluation of some thiazole-containing amino-acids and peptides. *European Journal of Medicinal Chemistry* **28**, 387–397.

- Vanderheyden, N., Wong, J. and Docampo, R. (2000). A pyruvate-proton symport and an H⁺-ATPase regulate the intracellular pH of *Trypanosoma brucei* at different stages of its life cycle. *Biochemical Journal* **346**, 53–62.
- Vanhamme, L., Paturiaux-Hanocq, F., Poelvoorde, P., Nolan, D.P., Lins, L., Van den Abbeele, J., Pays, A., Tebabi, P., Van Xong, H., Jacquet, A., Moguilevsky, N., Dieu, M. and Pays, E. (2003). Apolipoprotein L-I is the trypanosome lytic factor of human serum. *Nature* **422**, 83–87.
- Vannieuwenhove, S. (1988). Nifurtimox in late-stage arsenical-refractory gambiense sleeping sickness. *Bulletin de la Société de Pathologie Exotique* **81**, 650.
- Vanschaffingen, E., Opperdoes, F.R. and Hers, H.G. (1987). Effects of various metabolic conditions and of the trivalent arsenical melarsen oxide on the intracellular levels of fructose 2,6-bisphosphate and of glycolytic-intermediates in *Trypanosoma brucei*. *European Journal of Biochemistry* **166**, 653–661.
- Vansterkenburg, E.L.M., Coppens, I., Wilting, J., Bos, O.J.M., Fischer, M.J.E., Janssen, L.H.M. and Opperdoes, F.R. (1993). The uptake of the trypanocidal drug suramin in combination with low-density lipoproteins by *Trypanosoma brucei* and its possible mode of action. *Acta Tropica* **54**, 237–250.
- Verlinde, C.L.M., Hannaert, V., Blonski, C., Willson, M., Perie, J.J., Fothergill-Gilmore, L.A., Opperdoes, F.R., Gelb, M.H., Hol, W.G.J. and Michels, P.A.M. (2001). Glycolysis as a target for the design of new anti-trypanosome drugs. *Drug Resistance Updates* **4**, 50–65.
- Viode, C., Bettache, N., Cenas, N., Krauth-Siegel, R., Chauviere, G., Bakalara, N. and Perie, J. (1999). Enzymatic reduction studies of nitroheterocycles. *Biochemical Pharmacology* **57**, 549–557.
- Voogd, T.E., Vansterkenburg, E.L.M., Wilting, J. and Janssen, L.H.M. (1993). Recent research on the biological activity of suramin. *Pharmacological Reviews* **45**, 177–203.
- Voorheis, H.P. (1971). Amino-acid transport in *Trypanosoma brucei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**, 241–243.
- Voorheis, H.P. (1973). Studies on active-site of N1 amino-acid transport carrier of *T. brucei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **67**, 256–257.
- Voorheis, H.P. (1977). Changes in kinetic-behavior of threonine transport into *Trypanosoma brucei* elicited by variation in hydrogen-ion concentration. *Biochemical Journal* **164**, 15–25.
- Voorheis, H.P. (1980). Fatty-acid uptake by blood-stream forms of *Trypanosoma brucei* and other species of the Kinetoplastida. *Molecular and Biochemical Parasitology* **1**, 177–186.
- Wallace, L.J.M., Candlish, D. and de Koning, H.P. (2002). Different substrate recognition motifs of human and trypanosome nucleobase

- transporters. Selective uptake of purine antimetabolites. *Journal of Biological Chemistry* **277**, 26149–26156.
- Wallace, L.J.M., Candlish, D., Hagos, A., Seley, K.L. and de Koning, H.P. (2004). Selective transport of a new class of purine antimetabolites by the protozoan parasite *Trypanosoma brucei*. *Nucleosides Nucleotides & Nucleic Acids* **23**, 1441–1444.
- Walmsley, A.R., Barrett, M.P., Bringaud, F. and Gould, G.W. (1998). Sugar transporters from bacteria, parasites and mammals: structure-activity relationships. *Trends in Biochemical Sciences* **23**, 476–481.
- Wiemer, E.A.C., Terkuile, B.H., Michels, P.A.M. and Opperdoes, F.R. (1992). Pyruvate transport across the plasma-membrane of the blood-stream form of *Trypanosoma brucei* is mediated by a facilitated diffusion carrier. *Biochemical and Biophysical Research Communications* **184**, 1028–1034.
- Wiemer, E.A.C., Michels, P.A.M. and Opperdoes, F.R. (1995). The inhibition of pyruvate transport across the plasma-membrane of the blood-stream form of *Trypanosoma brucei* and its metabolic implications. *Biochemical Journal* **312**, 479–484.
- Wierenga, R.K., Swinkels, B., Michels, P.A.M., Osinga, K., Misset, O., Vanbeeumen, J., Gibson, W.C., Postma, J.P.M., Borst, P., Opperdoes, F.R. and Hol, W.G.J. (1987). Common elements on the surface of glycolytic-enzymes from *Trypanosoma brucei* may serve as topogenic signals for import into glycosomes. *EMBO Journal* **6**, 215–221.
- Wille, U., Schade, B. and Duszenko, M. (1998). Characterization of glycerol uptake in bloodstream and procyclic forms of *Trypanosoma brucei*. *European Journal of Biochemistry* **256**, 245–250.
- Witola, W.H., Inoue, N., Ohashi, K. and Onuma, M. (2004). RNA-interference silencing of the adenosine transporter-1 gene in *Trypanosoma evansi* confers resistance to diminazene aceturate. *Experimental Parasitology* **107**, 47–57.
- Xong, H.V., Vanhamme, L., Chamekh, M., Chimfwembe, C.E., Van den Abbeele, J., Pays, A., Van Meirvenne, N., Hamers, R., De Baetselier, P. and Pays, E. (1998). A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. *Cell* **95**, 839–846.
- Yardley, V. and Croft, S.L. (2000). A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. *International Journal of Antimicrobial Agents* **13**, 243–248.
- Yarlett, N. and Bacchi, C.J. (1988). Effect of DL- α -difluoromethylornithine on methionine cycle intermediates in *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **27**, 1–10.
- Zilberstein, D. (1993). Transport of nutrients and ions across membranes of trypanosomatid parasites. *Advances in Parasitology* **32**, 261–291.

Making Sense of the Schistosome Surface

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ABSTRACT

The syncytial cytoplasmic layer, termed the tegument, which covers the entire surface of adult schistosomes, is a major interface between the parasite and its host. Since schistosomes can survive for decades within the host bloodstream, they are clearly able to evade host immune responses, and their ability is dependent on the properties of the tegument surface. We review here the molecular organization and biochemical functions of the tegument, combining the extensive literature over the last three decades with recent proteomic studies. We have interpreted the organization of the tegument surface as bounded by a conventional plasma membrane overlain by a membrane-like secretion, the membranocalyx, with which host molecules can associate. The range of parasite proteins, glycans and lipids found in the surface complex is evaluated, together with the host molecules detected. We consider the way in which the tegument surface is formed after cercarial penetration into the skin, and changes that occur as parasites develop to maturity. Lastly, we review the evidence on surface dynamics and turnover.

1. INTRODUCTION

Schistosomes are platyhelminth parasites that represent a serious public health problem, infecting an estimated ~200 million people worldwide. Adult worms live in the bloodstream surrounded by all the components of the immune response, yet are able to survive for

decades. This means that they must possess sophisticated immune evasion strategies that are a match for the immune system. The surface of the parasite is covered by a cytoplasmic layer, the tegument, which is believed to be pivotal in promoting parasite survival via its role in immunoevasion. In addition, the tegument is an important site of nutrient uptake from the host. The purpose of this review is to summarize what we know about the organization and function of this remarkable structure.

1.1. External Morphology of the Adult Tegument

Mature male schistosomes, or blood flukes, are approximately 10 mm long and dorsoventrally flattened with their lateral margins curving ventrally and overlapping to form a gynaecophoric canal. The mature female is cylindrical, longer, and more slender than the male and is held in the gynaecophoric canal of the male. In cross section, the male/female pair spans about 1 mm. Both sexes possess an anterior, oral sucker, which opens into the alimentary tract and a ventral sucker located about 1 mm further back. Suckers are used for attachment to the blood vessel lining and to facilitate intravascular movement (Hockley, 1973).

Large tubercles or papillae are present on the dorsal surface of male *Schistosoma mansoni*, posterior to the ventral sucker. The sides of the tubercles are studded with prominent, rigid, intracellular spines composed of actin bundles (Cohen *et al.*, 1982). Elsewhere the worm surface is covered in evenly spaced pits and bears some spines and sensory papillae; it may be flattened or thrown into ridges depending on the state of contraction of the underlying muscles. The surface of the gynaecophoric canal is ridged and covered with small, irregularly arranged spines. Female worms possess comparatively few spines and their surface, while smoother and lacking large tubercles, is otherwise similar to the pitted and ridged surface of the males (Senft *et al.*, 1961; Silk *et al.*, 1969). Spines are most numerous on the posterior end of females (Hockley, 1973). Most work on the schistosome tegument has focused on *S. mansoni*; the tegumental morphology of other schistosome species is similar although some differences in topology and

ultrastructure have been reported (Inatomi *et al.*, 1969; Sobhon *et al.*, 1984).

1.2. Cell Biology of the Tegument

The tegument is one of the two major interfaces between the schistosome and its external environment, the other being the lining of the gut. In most features, the schistosome tegument, as illustrated in Figure 1, corresponds to the basic platyhelminth tegument reviewed by Threadgold (1984). Since the tegument lacks lateral membranes, its cytoplasm extends as a continuous unit, or syncytium, around the entire body (Morris and Threadgold, 1968; Smith *et al.*, 1969); it varies in thickness from 1 to 3 μm (Wilson and Barnes, 1974b). The tegumental cytoplasmic layer, lying beneath the surface membranes, contains small mitochondria and two different kinds of secretory inclusions called discoid bodies and multilaminar vesicles (Wilson and Barnes, 1974b). Discoid bodies are numerous in the adult worm; there

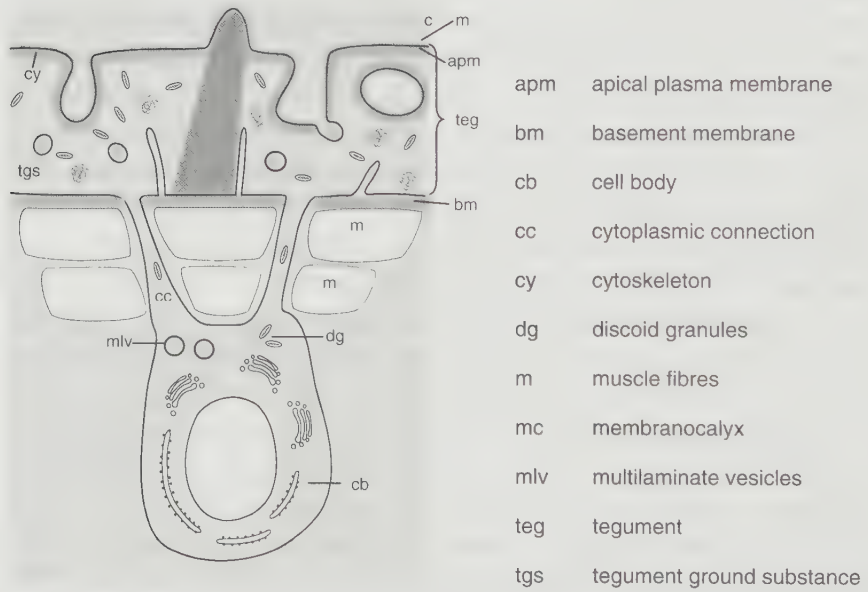


Figure 1 Diagram of the adult schistosome tegument and associated cell bodies.

are about 20-fold more of these versus multilaminate vesicles per μm^2 of tegumental cytoplasm (Wilson and Barnes, 1974b). They are approximately 40 nm by 200 nm in size, are surrounded by a single lipid bilayer and have a dense granular, mucopolysaccharide content (Wilson and Barnes, 1974b). Multilaminate vesicles are 150–200 nm in diameter and appear as a mass of tightly packed concentrically arranged membranes. The tegument cytoplasm is connected by numerous, slender, often tortuous, microtubule-lined, cytoplasmic processes to cell bodies (or cytons) that lie beneath the peripheral muscle layers. These contain the nucleus as well as endoplasmic reticulum, golgi complexes and mitochondria, and are occasionally multinucleate (Morris and Threadgold, 1968; Silk *et al.*, 1969). Proteins and carbohydrates for export are synthesized in the cytons and packaged into the secretory inclusions by numerous Golgi bodies for transit along the cytoplasmic connections to the tegument cytoplasmic layer (Smith *et al.*, 1969). Chemical disruption of microtubule function results in an accumulation of the secretory bodies in the cytons (Zhou and Podesta, 1989). Cytons may be widely separated in the intervening parenchyma by 5–20 μm but can be linked by intercellular bridges (Smith *et al.*, 1969; Wilson and Barnes, 1974b). Neither DNA nor RNA can be detected in the tegument syncytium by histochemical staining, indicating that all synthesis of proteins is confined to the cytons (Wheater and Wilson, 1976).

The major distinction between intravascular digenetic trematodes such as schistosomes and those occupying other habitats, such as the gut, is that the external tegument of blood flukes has a multilaminate appearance that is evident particularly when uranyl acetate is used as a fixation step for electron microscopy, additional to the conventional glutaraldehyde and osmium tetroxide (Hockley and McLaren, 1973); its unusual organization is considered to be an adaptation for survival in the bloodstream. Initially, the surface structure was described as a heptalaminate membrane, and several ways in which the multilaminate vesicles might contribute to its organization were suggested. However, the fact that the outer lamellae were only visualized by uranyl acetate treatment suggested that they were less stable, and hence materially different in composition, than the inner ones (Wilson and Barnes, 1974b). These and other observations led Wilson and

Barnes (1974a) to suggest that the seven-layered (heptalaminate) surface was in reality two closely apposed lipid bilayers in the form of a normal plasma membrane overlain by a membrane-like secretion. (The classical lipid bilayer unit membrane appears trilaminate in electron micrographs, so two lipid bilayers separated by a space would have a heptalaminate appearance.) Furthermore, they proposed that a multilaminate vesicle contributed its contents to form the outer bilayer when its bounding membrane fused with the tegument plasma membrane, in a conventional process of exocytosis. The contents of the multilaminate vesicle could thus be considered the membranous equivalent of the glycocalyx in animal cells, and in a second paper they proposed the term 'membranocalyx' for the outer bilayer (Wilson and Barnes, 1974a). The model of a tegument surface consisting of an inner apical plasma membrane and an outer secreted membranocalyx is adopted throughout this review to provide a context for interpretation of the data generated by the many investigations of tegument structure and function. It is implicit in the model that the secreted membranocalyx will ultimately be shed into the external environment, while a mechanism for removing the excess plasma membrane, contributed by the bounding membrane of the multilaminate vesicle, must operate exactly as in other secretory cells, most probably by internalization.

Like the invaginated apical tegumental membranes, the basal membrane evaginates as a system of narrow channels of variable length into the tegumental cytoplasm (Silk *et al.*, 1969). The surface invaginations and the basal membrane evaginations may be separated by as little as 0.25–0.5 μm of tegumental cytoplasm (Wilson and Barnes, 1974b). Such tegumental folds and pits are suggestive of a highly absorptive surface, but may also be necessary for tegumental flexibility (Hockley, 1973). The tegument is perforated sporadically, especially on the suckers and male gynaecophoric canal, by bulbous, possibly tactile, sensory structures bearing an apical cilium, firmly attached by septate desmosomes (Morris and Threadgold, 1968; Silk *et al.*, 1969; Smith *et al.*, 1969). The tegument is demarcated from the underlying musculature by a finely granular basal lamina or basement membrane (Silk *et al.*, 1969).

As postulated above, tegumental products are presumably shed into the host bloodstream and antibodies against tegumental proteins, and

carbohydrates can be detected in the serum of infected animals (Brindley and Sher, 1987; Cutts and Wilson, 1997; Koster and Strand, 1994a; Nyame *et al.*, 1996; Remoortere *et al.*, 2000; van Remoortere *et al.*, 2001). However, since adult tegumental epitopes may be shared with other life stages (such as the very immunostimulatory egg), it remains unclear whether the tegument itself is a strong primary focus of anti-parasite immunity. Indeed, adult worms recovered from infected domestic or wild hosts exhibit no evidence of a sustained immunological attack focused on the tegument. While antibody to parasite antigens can be demonstrated on the surface of worms recovered from the bloodstream, much of this appears bound via the Fc domain making the antibody unavailable for productive interaction, e.g. with complement components or immune effector cells (Sogandares-Bernal, 1976; Kemp *et al.*, 1977, 1978, 1980; Bickle and Ford, 1982; Loukas *et al.*, 2001). Furthermore, using scanning electron microscopy, hardly any host cells are seen adhering to the tegument of schistosomes freshly recovered from the bloodstream of their hosts (Kruger and Wolmarans, 1990). A few leukocytes and platelet-sized cells are observed on the tegument of some parasites (Kruger and Wolmarans, 1990). Many mechanisms for evading destruction in immunocompetent hosts have been proposed and several of these involve the tegument, including rapid tegument turnover, the acquisition of masking host antigens and the loss of surface antigenicity with maturation.

1.3. Formation of the New Tegument within the Mammalian Host

The double-bilayered tegumental surface of schistosomes is specific to the intra-mammalian-stage parasites. When cercariae, infectious for mammals, emerge from infected snail intermediate hosts, their tegument is bounded by a single trilaminar membrane, which is covered by a thick, fibrillar glycocalyx. During host penetration, first the cercarial glycocalyx, and later the tegumental outer membrane, is shed. This latter process has been observed in parasite sections by electron microscopy and, in whole animals, by tracking the appearance and

movement of apical tegumental membrane proteins (Hockley and McLaren, 1973; Torpier *et al.*, 1979b; Brouwers *et al.*, 1999; Skelly and Shoemaker, 2001). Coincident with transformation, multilaminate vesicles migrate from the cytons into the tegumental cytoplasm and fuse at the surface. At this time, the outer coat has a patchy multilipid bilayer covering, and these lipid depositions resolve and coalesce to form the outer lipid bilayer (the membranocalyx) which overlays the inner lipid bilayer (the apical plasma membrane) (Figure 1).

As noted earlier, the adult tegument contains two major types of secretory bodies—multilaminate vesicles and discoid bodies. Discoid bodies are present in all parasitic flatworms, while multilaminate vesicles are present only in the blood flukes. This has led to the suggestion that discoid bodies are the precursors of the inner apical membrane, whereas the multilaminate vesicles are the precursors of the membranocalyx (Zhou and Podesta, 1989). However, discoid bodies are not seen in schistosomula until well after the new tegument is synthesized (Hockley and McLaren, 1973; Dorsey and Cousin, 1995). This implies that multilaminate vesicles alone can form both the new apical tegumental membranes. While this observation does not rule out a role for discoid bodies in the later maintenance or modification of the tegumental membranes, there is evidence that discoid bodies are transport vesicles that simply dissolve to release their contents into the tegumental cytoplasm (Wilson and Barnes, 1974a). The released material can be detected as blobs of mucopolysaccharide by electron microscopy (Wilson and Barnes, 1974a). Such depositions could enhance the biophysical properties of the tegument, serving to cushion it against compression and abrasion as the parasites migrate within the vasculature. This hypothesis precludes a role for discoid bodies in tegumental membrane biogenesis.

1.4. Surface Composition

The molecular composition of the outer surface membranes of intramammalian-stage parasites has been the subject of much work. There is great interest in identifying and characterizing surface molecules to gain a better understanding of the biochemistry of the host-interactive

parasite tegument. In addition, such exposed molecules might serve as important vaccine targets to elicit protective immunity. A series of recent investigations has used proteomics to characterize, by direct analysis, the composition of the parasite host interface (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006). This review attempts to integrate this new information with the large literature base that deals with the characterization of individual surface molecules.

The review focuses on molecules integral to, or associated with, the two bilayers at the host parasite interface. A freeze-fracture study of the tegumental surface of adults revealed that the highest density of intramembranous particles (representing proteins or molecular complexes) exists in the membranocalyx (Hockley *et al.*, 1975; Torpier *et al.*, 1979b). The observation is paradoxical since, as already noted, the membranocalyx is less stable than the underlying plasma membrane suggesting that it contains fewer structural components. The parasite surface has long been known to be chimerical since host molecules have been shown to be associated with it. Therefore, the derivation of the intramembranous particles seen in the membranocalyx (host or parasite or both) is unresolved. In this review, we first describe what is known about the proteins, carbohydrates and lipids of parasite origin, and then examine the nature of those host molecules reported to be associated with the tegument surface.

2. PARASITE SURFACE PROTEINS

We must first define the criteria that have been used to designate a protein as being at the parasite surface and introduce several caveats pertinent to such designations. First, many molecules have been identified as being tegumental, but this does not mean that they are linked to the outer membranes. The glycolytic enzyme phosphofructokinase is a case in point; this enzyme is widely distributed in the adult tegument, but is not found within or external to the surface double bilayer (Mansour *et al.*, 2000). Second, molecules reported to be at the surface of one intravascular life stage may not remain there at another stage. We draw particular attention to reports involving young schistosomula

derived from cercariae that have been cultured for up to three hours. The transformation of cercariae into schistosomula is multifaceted and involves dramatic morphological and biochemical changes, including radical alterations at the surface that were outlined earlier. We speculate that many of the molecules reported to be at the surface of such three-hour schistosomula may be only transiently found here as a result of the tegumental membrane rearrangements that are part of cercarial transformation. Third, proteins detected at the parasite surface from an analysis of parasite *sections* may, in fact, not be exposed in the living parasite. Finally, we note that adult tegumental tubercles, in particular, may be prone to damage during parasite recovery and handling. Thus proteins reported to be exclusively located at such sites may have only become exposed due to abrasion of the overlying membranocalyx and plasma membrane in the area.

With these caveats in mind, there are numerous reports of parasite proteins exposed at the surface of various schistosome species, although in some cases this localization is disputed. Proteins reported to be at the surface belong to several classes and include enzymes such as alkaline phosphatase, acetylcholinesterase, a 27-kDa esterase, glutathione *S*-transferases (GST), proteases (m28) and the glycolytic enzymes, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and triose phosphate isomerase (TPI). Additional, different enzyme activities have been detected at the tegument surface, while the enzymes themselves have not yet been identified (e.g. leucine aminopeptidase, sphingomyelinase). Other proteins detected at the host-parasite interface include those involved in the import of nutrients such as glucose and amino acids from the host vasculature (e.g. schistosome glucose transporter protein 4 (SGTP4) and schistosome amino acid permease 1 (SPRM1)). In addition, receptors or receptor homologs (acetylcholine receptor (AChR), *S. mansoni* receptor serine threonine kinase-1 (SmRK-1), low-density lipoprotein (LDL) receptor, Fc receptor/paramyosin) have been reported to be present at the surface, including a collection of putative receptors for components of the host's complement cascade (e.g. C2, C3, C8 and C9). Finally, there are reports of structural proteins or proteins of presently unknown function at the surface (Sm23, A1.12/9, spine glycoprotein, Sm200). These proteins are listed in Table 1.

Table 1 Schistosome proteins reported in the literature as being at the host-parasite interface. Results from conventional studies are compared with three recent proteomic studies, Ref #1: Van Balkom *et al.*, 2005; Ref #2: Braschi *et al.*, 2006; Ref #3: Braschi and Wilson, 2006

Molecule	Description	Evidence for surface location	Life stage examined	Adult tegument proteome analysis		
				Ref #1	Ref #2	Ref #3
Enzymes				Detached tegument	Membrane-enriched preparation	Available for biotinylation
	Alkaline phosphatase	65-kDa GPI-linked enzyme, tetramer	Somula, adult	No	Yes	Yes
	ATP-diphosphohydrolase	63-kDa enzyme	Adult	Yes	Yes	Yes
	Nucleoside-diphosphatase	Uncharacterized	Adult	Yes	Yes	Yes
	Acetylcholinesterase	GPI-linked dimer of 110 and 76-kDa subunits	Somula, adult	No	No	No
Esterase	27-kDa non-specific esterase	IF	Adult	No	No	No
m28 (elastase)	GPI-linked, 28-kDa serine protease	PIPLC release	Somula, lung worm, adult	No	No	No
Leucine aminopeptidase	Uncharacterized	Surface enzyme activity	Adult	No	No	No

(Continued)

Table 1 (Continued)

Molecule	Description	Evidence for surface location	Life stage examined	Adult tegument proteome analysis		
				Ref #1	Ref #2	Ref #3
Triose phosphate isomerase Glyceraldehyde-3-phosphate dehydrogenase Nutrient transporters SGTP4	28-kDa glycolytic enzyme	IF	Adult	No	Yes	No
	37-kDa glycolytic enzyme	IF	Somula	No	Yes	No
	55-kDa facilitative diffusion glucose transporter	IF, immuno-EM, surface labeling	Somula, adult	No	No	No
SPRM11c	55-kDa amino acid transporter	IF, surface labeling	Adult	No	No	No
	66-kDa TGFβ receptor	IF, surface labeling	Adult	No	No	No
Receptors Receptor serine threonine kinase homolog (SmRK-1) Acetylcholine receptor Low-density lipoprotein (LDL) receptors Complement C2 receptor (CRIT)	Uncharacterized	Receptor ligand binds to surface	Adult	—	—	—
	No consensus	Labeled LDL binds to surface	Somula, adult	—	—	—
	32-kDa C2-binding protein	Immuno-EM	Adult	No	No	No

Complement C3 receptor	130-kDa C3-binding protein	Conflicting C3/anti-C3 binding data	—	—	—
Paramyosin (Complement C8, C9 receptor (SCIP-1) and Fc receptor)	97-kDa major muscle protein	IF, Immuno-EM, PIPLC release, Conflicting IF reports and conflicting reports of Ig at the parasite surface	No	Yes	No
Structural proteins					
Sm23	23-kDa tetraspanin antigen, some GPI-linked	IF	No	Yes	No
Spine glycoprotein Sm200	170-kDa glycoprotein GPI-linked, 200-kDa glycoprotein	IF IF, PIPLC release	— No	— No	— Yes

Note: ' ' indicates that the protein's sequence is unknown and therefore it cannot be detected by proteomic analysis.
IF, immunofluorescence; PIPLC, phosphatidylinositol phospholipase C

Several criteria have been used to determine the surface localization of parasite proteins. In many instances, immunofluorescent, immunohistochemical or immuno-EM analysis was employed to localize specific proteins directly at the parasite surface. Sometimes, evidence for exposure at the parasite surface derives from an ability to label the molecules on intact parasites. In a number of cases, molecules are reported to be at the surface since they can be released from whole parasites by enzymatic treatment (using, e.g., phosphatidylinositol phospholipase C (PIPLC), which cleaves exposed glycosyl phosphatidyl-inositol (GPI)-linked, membrane-associated molecules). On other occasions, the enzymatic activity of a protein can be detected at the surface. Some surface proteins have also been demonstrated to provide a measure of protection to experimental animals following vaccination, suggesting that they are exposed to immunological effectors. Finally, tegument-enriched preparations have been subjected to proteomic analysis to identify their protein composition. These proteomic studies (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006) used a freeze thaw vortex method (Roberts *et al.*, 1983) to detach the adult tegument from the underlying worm bodies; thereafter their respective methods diverged. In one study, the detached whole tegument was pelleted, washed and its composition analyzed in comparison with the denuded bodies, similarly treated (van Balkom *et al.*, 2005). Database searching with the numerous fragmentation spectra identified following mass spectrometry (MS) produced 43 protein identities unique to the detached tegument, 207 unique to the denuded bodies and 179 shared between the two. This large number of common proteins is not unexpected since the tegumental proteins are synthesized in cytons that lie beneath the peripheral muscle bundles. This means that tegumental cytons will be included in the 'denuded worm' fraction. The unpurified nature of the starting material does not permit inferences to be made about the location of identified constituents on the surface or within the tegumental cytoplasm.

In a second study (Braschi *et al.*, 2006), the detached tegument alone was analyzed, but the released material was first enriched for surface membranes by sucrose gradient centrifugation, using alkaline

phosphatase as a marker (Roberts *et al.*, 1983). The membrane fraction was then subjected to sequential extractions with reagents of increasing solubilizing power, namely TRIS, urea thiourea and urea thiourea CHAPS sulfobetaine to leave a final, insoluble pellet enriched for transmembrane proteins. All fractions were processed and analyzed by MS. The results obtained permitted inferences to be made about the location and relative abundance of proteins associated with the tegument surface bilayers, but not about their accessibility to the external environment.

The above question of accessibility, of considerable relevance to studies on immunity and vaccine development, has been tackled in a third proteomic study (Braschi and Wilson, 2006). Live adult worms were incubated with two forms of an impermeant biotinylation reagent. Their teguments were detached by the freeze thaw method, pelleted and subjected to five cycles of extraction with solvents of increasing strength to release the proteins. Any biotinylated constituents were then recovered with streptavidin affinity beads and resolved by 1D SDS-PAGE. Biotinylated gel bands were excised, trypsinized and subjected to LC and tandem MS. The study revealed 13 proteins (four derived from the host and nine from the parasite) that were biotinylated by the long-form reagent, while the same 13, plus a further 15, were labeled by the short-form reagent. In the review of tegumental proteins below, pertinent information from all of these proteomic studies is provided in each section, while a resume of other major proteomic findings is dealt with in Section 2.7.

Table 1 lists some features of the molecules that have been reported in the literature to be present at the schistosome surface. In the table, the criteria used for determining the surface localization of each molecule are noted. Whether the molecules have been detected in the recent proteomic analyses of tegumental composition using MS is also indicated. Clearly, detection of a molecule in the tegumental membranes by MS represents the strongest corroborative evidence for its location at the host-parasite interface. An inability to detect a previously characterized tegumental protein in such studies may reflect its absence or its lower relative abundance at the surface.

2.1. Surface Enzymes

2.1.1. Phosphatases

Over 45 years ago, *alkaline phosphatase* activity was histochemically located in the tegument, gastrodermis and reproductive structures of adult worms (Dusanic, 1959; Halton, 1967). Alkaline phosphatase activity was detected on the external surface of the tegument and in the cytoplasmic tubules connecting cytons with the outer tegument (Morris and Threadgold, 1968). An even distribution of alkaline phosphatase activity covering all areas of the female tegument was reported (Ninno-Smith and Standen, 1963). In male worms, only the dorsal tegument exhibits activity with virtually no activity at the ventral surface, except for small amounts in the ventral sucker region (Ninno-Smith and Standen, 1963; Morris and Threadgold, 1968). A tegument-enriched fraction obtained by saponin treatment of adult *S. mansoni* contained about 70% of the alkaline phosphatase activity in both sexes (Cesari, 1974).

Since the alkaline phosphatase activity is predominantly associated with the tegumental membrane fraction, it has been used by several workers as a marker for surface membrane isolation (Roberts *et al.*, 1983; Payares *et al.*, 1985b). A monoclonal antibody against schistosome alkaline phosphatase clearly binds to the tegument in adult parasite sections (Pujol *et al.*, 1990). Additionally, an enriched schistosomula outer tegumental membrane preparation has alkaline phosphatase activity (Levi-Schaffer *et al.*, 1984). Since alkaline phosphatase activity is detected in the material released from cultured schistosomula following PIPLC treatment, this suggests that some of the enzyme is phosphatidyl inositol (PI) anchored at the surface (Espinoza *et al.*, 1988).

The *S. mansoni* alkaline phosphatase has been purified from Triton X-100 extracts of adult parasites following ConA agarose affinity chromatography and gel filtration; it is a glycoprotein of Mr 260 000, composed of four enzymatically inactive glycosylated subunits of Mr 65 000 (Payares *et al.*, 1984). There was no difference in the molecular size of egg, cercaria, schistosomulum and purified adult worm alkaline phosphatase. While the adult alkaline phosphatase could be

radiolabeled on the parasite surface, the enzyme was reported to be poorly accessible to labeling reagents (Payares *et al.*, 1984). In contrast, the enzyme was labeled and immunoprecipitated with ease when three-week-old worms or three-day-old lung worms were used (Payares *et al.*, 1984). It was suggested that perhaps the enzyme becomes more 'buried in the tegumental membrane network' as the parasite matures (Payares *et al.*, 1984).

The host is exposed to parasite alkaline phosphatase and an immune response is generated against the molecule; most infected humans tested (14 of 15, 93%) show a clear antibody response against the enzyme (Pujol and Cesari, 1990). The anti-alkaline phosphatase antibody response is detected at around 50 days post-infection, after the parasites have matured. Antibody from chronically infected mice can partially inhibit tegumental alkaline phosphatase activity (Cesari *et al.*, 1981; Pujol and Cesari, 1990; Fallon *et al.*, 1994).

The question of whether surface enzymes like alkaline phosphatase are synthesized by the parasite or are acquired from the host has been raised. For alkaline phosphatase at least, the host enzyme had a lower molecular size activity relative to its worm counterpart (Payares *et al.*, 1984). Because of this and since infected mice sera show no reactivity to host alkaline phosphatases, it is clear that the tegumental alkaline phosphatase of *S. mansoni* is synthesized by the parasite and is not acquired from host tissue (Pujol and Cesari, 1990). Absolute proof that the parasites can produce their own alkaline phosphatase now exists; a homolog can be identified in the *S. mansoni* genome database (Verjovski-Almeida *et al.*, 2003). Recent proteomic analysis confirms that alkaline phosphatase is found in the schistosome surface membranes (van Balkom *et al.*, 2005; Braschi *et al.*, 2006) and is available for surface biotinylation (Braschi and Wilson, 2006).

Other workers have detected phosphatase activities at the parasite surface but have not isolated the responsible molecule(s). For example, an *ATP diphosphohydrolase* (apyrase) was reported to be present on the surface of the tegument of adult *S. mansoni* (Vasconcelos *et al.*, 1993). The enzyme activity had broad substrate specificity (being capable of hydrolyzing ATP, ADP and other nucleotides) and was identified in an adult tegument fraction (Vasconcelos *et al.*, 1993). EM analysis identified electron-dense lead phosphate deposits on the

outer surface of adult parasites upon hydrolysis of ATP or ADP and the production of inorganic phosphate (Vasconcelos *et al.*, 1993, 1996).

Incubating worms with the ATP analog, fluorosulfonylbenoyl adenosine, substantially inhibits ATPase and ADPase activity indicating that the ATP-binding site is accessible to the analog from the worm's external surface (Torres *et al.*, 1998). Immunoblotting of an *S. mansoni* tegument preparation revealed a 63-kDa ATP diphosphohydrolase and heterologous anti-apyrase antisera bind to the parasite surface (Vasconcelos *et al.*, 1996).

Earlier, *nucleosidediphosphatase* (NDPase) activity was localized cytochemically, using electron microscopy to the plasma membranes of the tegumental invaginations (the pits), but not the exposed surface, of adult *S. mansoni* (Bogitsh and Krupa, 1971). Hydrolysis of uridine diphosphate (UDP), inosine diphosphate (IDP) and guanosine diphosphate (GDP) as well as the triphosphates was detected. The NDPase activity required the presence of Mn^{2+} ions. While monophosphate esters and thiamine pyrophosphate could also be hydrolyzed, this activity was independent of Mn^{2+} suggesting the presence of at least two phosphatase enzymes of differing specificities at the parasite surface (Bogitsh and Krupa, 1971). Proteomic analysis of tegument preparations identifies such a protein in the tegumental membranes but classifies it more correctly as a *phosphodiesterase* by composition (Braschi *et al.*, 2006). This molecule is biotinylated at the surface (Braschi and Wilson, 2006).

The role of tegumental phosphatases at the host-parasite interface is likely related to purine recovery and/or the regulation and transport of phosphate ions across the tegument (Pujol *et al.*, 1990). Surface phosphatases may also aid in the evasion of host defense mechanisms through the hydrolysis of ADP and/or ATP released by activated platelets and cytotoxic T lymphocytes (Torres *et al.*, 1998).

2.1.2. Esterases

More than 35 years ago esterase activity was identified histochemically in various schistosome tissues including the adult tegument

(Fripp, 1967a). More recently, some of the molecules responsible for this surface esterase activity have been identified.

Acetylcholinesterase (AChE). Acetylcholine, an important neurotransmitter, is hydrolyzed by the action of AChEs. AChE activity is associated, in many organisms including schistosomes, with the neuromuscular system (Pax *et al.*, 1984). Activity is also detected in a schistosomula preparation enriched for the external membranes (Levi-Schaffer *et al.*, 1984). An antibody raised against a heterologous AChE (from the electric eel) stained the outer membranes of adults and schistosomula, supporting the notion that some AChE activity is exposed at the surface (Tarrab-Hazdai *et al.*, 1984). Likewise, monoclonal antibodies (MAbs) raised against *S. mansoni* AChE stain the surface of 24-hour schistosomula (Espinoza *et al.*, 1995). Immunolocalization experiments using electron microscopy and frozen sections of schistosomula showed a clear surface localization of immunogold particles (Espinoza *et al.*, 1991b). Binding was also observed to membranous bodies within the tegument and in the peripheral muscle (Espinoza *et al.*, 1991b).

One characteristic of AChEs is their rich molecular polymorphism leading to the presence of multiple molecular forms in different developmental stages (Espinoza *et al.*, 1995). Such differences likely reflect post-translational modification, particularly glycosylation. Most MAbs generated against the schistosome AChE detect the protein in cercariae, schistosomula and adult parasite preparations by western analysis, demonstrating an antigenic continuity between adult and larval stages (Espinoza *et al.*, 1995). The surface protein is thought to exist as a dimeric globular (or G2) form consisting of 110 kDa and 76 kDa subunits with additional, larger multimeric forms associated with internal tissues (Camacho *et al.*, 1994). Two polypeptide bands of 110 and 76 kDa are immunoprecipitated from extracts of adults of three schistosome species: *S. mansoni*, *Schistosoma haematobium* and *Schistosoma bovis* (Camacho *et al.*, 1994). The internal form of AChE, unlike the tegumental enzyme, is reported to bind to heparin (Arnon *et al.*, 1999).

Enzyme analysis suggests that about 50% of the total parasite AChE is present at the surface of intact 24-hour schistosomula (Espinoza *et al.*, 1995). Differences in the relative amounts of AChE

activity in the tegument of three schistosome species have been reported. *S. haematobium* teguments carried 20 times and *S. bovis* almost 7 times the activity of *S. mansoni* teguments (Camacho *et al.*, 1994). It has been suggested that tegumental AChE is the vital target of the schistosomicidal drug, metrifonate (Camacho *et al.*, 1994), and *S. haematobium* is more susceptible to this drug *in vivo*, being killed more readily than *S. mansoni* or *S. bovis* (Marshall, 1987).

A substantial amount of the enzyme is PI anchored, since it can be released from living parasites by PIPLC with no apparent impairment in parasite viability (Espinoza *et al.*, 1988, 1991a; Camacho *et al.*, 1996). Cleavage of AChE by PIPLC is reported to induce new AChE biosynthesis, since AChE levels increase in treated parasites (while no increase in the synthesis of control proteins is observed) (Espinoza *et al.*, 1991a).

The host is exposed to parasite AChE and an immune response is generated against the molecule: most infected mice (19 of 22, 86%) show a clear antibody response against the enzyme by ELISA while controls are negative. Similarly, most infected humans (14 of 16, 87%) recognize the parasite enzyme in radio-immuno assays (Espinoza *et al.*, 1991b). Note that this anti-AChE response may not be primarily or initially generated against the tegumental enzyme but rather against AChE released from another life stage (e.g. the egg).

An AChE from *S. haematobium* (*ShAChE*) has been cloned and characterized (Jones *et al.*, 2002). Degenerate primers derived from conserved AChEs were used to obtain an AChE DNA fragment by PCR and this fragment was used to isolate a full-length clone. The predicted open reading frame encodes a protein of 689 amino acids, including a putative signal sequence of 25 residues (Jones *et al.*, 2002). While the estimated Mr of *ShAChE* is ~80 kDa, recombinant *ShAChE* expressed in *Xenopus* oocytes migrates as a single band of ~116 kDa following SDS-PAGE, suggestive of post-translational modification. *ShAChE* possesses five putative *N*-glycosylation sites. Sequence identities between *ShAChE* and homologs from *Caenorhabditis elegans*, *Drosophila melanogaster* or humans are similar and vary from 29% to 33% (Jones *et al.*, 2002). Despite evidence that the native enzyme is GPI anchored, the sequence at the C-terminus of *ShAChE* does not conform to the current consensus for GPI modification (Massoulie

et al., 1998). The enzyme, expressed in *Xenopus* oocytes, shows conventional substrate specificity and sensitivity to established inhibitors of AChEs, including metrifonate (Jones *et al.*, 2002). Polyclonal antibodies, raised against a synthetic *Sh*AChE peptide, confirmed the localization of the enzyme to the worm surface and underlying muscle tissue. Confocal microscopy of intact live worms showed clear labeling of the worm surface alone (Jones *et al.*, 2002). AChE is detected in the tegumental membranes in one study of the tegument proteome (S. Braschi, personal communication) but not in others (van Balkom *et al.*, 2005).

The roles of AChEs in bacteria, plants and outside of the nervous system, in 'non-cholinergic' locations of complex organisms such as on the vertebrate red blood cell membrane, have not been elucidated (Mudad and Telen, 1996; Dave *et al.*, 2000). In the case of other parasitic helminths, roles for AChEs in altering cell membrane permeability or acting as anti-coagulants, or to increase nutrient availability, have all been proposed (Lee, 1996). In some nematode species, a substantial fraction of the AChE is secreted (Lee, 1996). Exposure of *S. haematobium* to acetylcholine *in vitro* alters the rate of glucose uptake, an effect that is concentration dependent and can be eliminated by inhibitors of the surface AChE or an acetylcholine receptor (Camacho and Agnew, 1995). The physiological significance of this finding is unclear. Since a number of adhesion proteins display sequence similarity with cholinesterases, this has led to the suggestion that some AChEs may constitute a novel class of adhesion molecules (Botti *et al.*, 1998). Despite such speculation, the precise physiological function of either AChE or an acetylcholine receptor (AChR, see Section 2.2.2) at the schistosome surface remains unknown.

27-kDa esterase. Non-specific esterase activity has been reported in the tegument and cytons using histochemical techniques (Wheater and Wilson, 1976). Furthermore, in passive transfer experiments, serum from a schistosome-infected rabbit acted synergistically with the schistosomicidal drug praziquantel (PZQ) to debilitate adult worms. A 27-kDa antigen was identified as being one target of this serum (Doenhoff *et al.*, 1988) and possessed non-specific esterolytic activity. Antibody against the protein stains the surface of freshly perfused,

adult parasites only slightly, but surface staining is dramatically increased following PZQ treatment. This result suggests that the action of the drug exposes the 27-kDa molecule (Doenhoff *et al.*, 1988) although whether it implies an internal location is unclear. No esterase homolog with this Mr was detected in any proteomic studies (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

2.1.3. Glutathione S-Transferase

A 20-kDa antigen (P28 or GST 28) was purified from an adult worm homogenate and used to immunize experimental animals (Balloul *et al.*, 1987). These animals exhibited significant protection from experimental infection (40–72%) (Balloul *et al.*, 1987). Sera from the animals also provided passive protection against infection (Balloul *et al.*, 1987). This protective antigen was identified as a GST. GSTs represent one of the major detoxification systems found in helminths (Brophy and Barrett, 1990). GST 28 has been localized in the tegument (and in the parenchyma and protonephridia) (Taylor *et al.*, 1988). Additionally, GST 28 was immunoprecipitated from iodinated worms leading to the suggestion that the protein has some association with the surface or is present among the excreted–secreted metabolites (Balloul *et al.*, 1987). However, this surface localization of GSTs in schistosomes is disputed. GST 28 was not detected in the tegument by other workers (Holy *et al.*, 1989). Furthermore, the protein was immunolocalized throughout the parenchyma of *S. mansoni* schistosomula but was not seen on the surface of living parasites (Balloul *et al.*, 1987). Finally, in later vaccine experiments, recombinant GST has not consistently yielded a protective immune response to challenge infection (Schechtman *et al.*, 2001).

In one proteome study, no glutathione transferases were identified in the parasite surface membranes by MS, but were detected in tegument cytosolic fractions (Braschi *et al.*, 2006). In another study, GST28 was detected in both the tegument fraction and in the parasite carcass (van Balkom *et al.*, 2005).

2.1.4. *Proteases*

m28. Transformation of cercariae into schistosomula is accompanied by the release of a soluble 28-kDa serine protease (s28, elastase) from the acetabular glands (Ghendler *et al.*, 1994). Schistosomula express on their surface a similar (and possibly identical) 28-kDa serine protease (m28) that can be immunoprecipitated with anti-s28 antibodies and that exhibits similar inhibitor sensitivities to s28 (Ghendler *et al.*, 1996). m28 was eluted from the schistosomula tegumental membranes with NP-40 and purified to homogeneity. Treatment with PI-PLC followed by hydroxylamine released m28 from the parasites. Anti-cross-reactive determinant antibodies (which recognize a neo epitope exposed in glycosyl phosphatidyl inositol-containing molecules cleaved by PIPLC) bind to purified m28 (Ghendler *et al.*, 1996). These data suggest that some of the m28 molecules are anchored to the tegumental membrane of schistosomula via glycosyl phosphatidyl inositol. Additionally, m28 was detected antigenically on lung-stage and adult worms (Ghendler *et al.*, 1996). Note that an adult worm tegumental extract contains inhibitory activity to the 28-kDa serine protease (Ghendler *et al.*, 1994). However, m28 elastase has not been identified in any of the tegument proteome reports (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006), nor have transcripts been detected in any life cycle stage other than the germ balls that develop into cercariae within daughter sporocysts (P. Ashton, personal communication).

Leucine aminopeptidase activity was detected histochemically in the dorsal and lateral tegument of adult male *Schistosoma rodhaini* and to a lesser extent throughout the tegument of females (Fripp, 1967b). The enzyme was hypothesized to aid in the production of free amino acids which could then be taken up across the outer body surface (Fripp, 1967b). However, it was not detected in the tegument or cytons of *S. mansoni* by similar techniques (Wheater and Wilson, 1976). No clear leucine aminopeptidase homolog has been identified solely in tegument-enriched fractions of *S. mansoni* by proteomics although an aminopeptidase homolog has been reported in both the parasite tegument and denuded bodies in one study (van Balkom *et al.*, 2005).

2.1.5. Glycolytic Enzymes

Perhaps surprisingly, normally cytosolic, glycolytic enzymes such as TPI and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported to be associated with the parasite surface (Goudot-Crozel *et al.*, 1989; Harn *et al.*, 1992).

TPI. TPI is a dimeric enzyme that converts glyceraldehyde-3-phosphate to dihydroxyacetone phosphate, a key reaction in glycolysis. Schistosome TPI was first identified as a target of a monoclonal antibody (M.1), generated from mice immunized with a detergent extract of schistosomula (Harn *et al.*, 1992). Passive transfer of the M.1 antibody confers resistance (41–50%) to mice following cercarial challenge (Harn *et al.*, 1992). Immunolocalization suggests that TPI is not only widely distributed in the internal tissues of the parasite, but also on or near the surface membrane of the adults (Harn *et al.*, 1992). The cDNAs encoding TPI from *S. mansoni* and *Schistosoma japonicum* have both been cloned and functionally characterized (Shoemaker *et al.*, 1992; Sun *et al.*, 1999).

GAPDH. In an epidemiological study in Bahia, Brazil, human resistance to *S. mansoni* infection was associated with IgG reactivity to a 37-kDa schistosomula surface antigen (Dessein *et al.*, 1988). A cDNA clone encoding this antigen was identified as glyceraldehyde-3P-dehydrogenase (GAPDH) (Goudot-Crozel *et al.*, 1989). GAPDH is best known for its enzymatic conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate during glycolysis. A polyclonal antiserum raised against the recombinant schistosome enzyme was used for immunofluorescent localization of the protein. Strong homogeneous fluorescence was associated with the surface of fresh schistosomula (Goudot-Crozel *et al.*, 1989). However, the predicted sequence of the cloned cDNA does not contain a typical transmembrane domain nor a terminal hydrophobic tail commonly found in most proteins anchored through PI (Goudot-Crozel *et al.*, 1989). Some, but not all, formulations of recombinant antigenic peptides derived from the molecule have proven to be protective following vaccination, yielding a 27–38% reduction in worm burdens (Argiro *et al.*, 1999, 2000a, b).

Both TPI and GAPDH have been identified in proteomic analyses of the tegument and tegumental membranes of adult worms, together

with enolase, fructose biphosphate aldolase and phosphoglycerate mutase (van Balkom *et al.*, 2005; Braschi *et al.*, 2006). Of these five glycolytic enzymes, GAPDH appeared most tightly bound to the tegument plasma membrane (Braschi *et al.*, 2006).

2.1.6. Other Enzyme Activities Associated with the Surface

Studies involving the tracking of fluorescent lipid analogues of ceramide and sphingomyelin have led to the suggestion that a neutral *sphingomyelinase* is located at the adult surface (Redman *et al.*, 1997).

2.2. Receptors or Receptor Homologs

2.2.1. Receptor Serine Threonine Kinase Homolog (*SmRK-1*)

A cDNA clone encoding a receptor serine threonine kinase homolog from the transforming growth factor β (TGF β) super family, designated SmRK-1, was identified in *S. mansoni* (Davies *et al.*, 1998). The clone was obtained by probing an adult male cDNA library with a PCR product obtained using degenerate primers. Antisera generated against recombinant SmRK-1 detected the 594 amino acid protein in biotinylated surface extracts (as well as in the parasite carcass). When used for immunofluorescence localization, the antisera revealed a punctate staining pattern corresponding to the tubercles found on the dorsal surface of male worms. As noted earlier, localization only to the tubercles may suggest surface abrasion at these protrusions during specimen preparation to permit antibody accessibility.

SmRK-1 exhibits 58% sequence identity with kinase domains of other receptors in the TGF β family and contains a conserved glycine-serine motif. The protein is detected only after the second week of infection of the mammalian host and only in males. It has been hypothesized to function in signal transduction across the surface membrane once the parasites have reached the hepatic portal circulation and perhaps in male female interactions. Equally the receptor could be involved in internal parasite signaling in such processes as tissue differentiation. In support of a signaling role for

SmRT1, human TGF β binds and activates the receptor when it is expressed in a heterologous system (Beall and Pearce, 2001). Additionally, SmRK-1 can be detected in its phosphorylated form (Davies *et al.*, 1998). Smad proteins are essential intracellular signal transducers of the TGF β super family and many homologs can be identified in the parasite transcriptome (Verjovski-Almeida *et al.*, 2003). Two Smad homologs, designated SmSmad1 and SmSmad2, have been characterized in *S. mansoni*, and SmRK-1 can interact with both (Beall *et al.*, 2000; Osman *et al.*, 2001). SmRK-1 has not been identified in tegumental fractions by proteomic analysis (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

2.2.2. *Acetylcholine Receptor*

Acetylcholine receptors (AChRs) are divided into two main types, designated nicotinic and muscarinic. The snake venom α -bungarotoxin (α -BTX) is a ligand of nicotinic AChRs. BTX binds to the dorsal surface of adult male schistosomes (Camacho *et al.*, 1995), indicating the presence of an AChR at this site. AChR could not be detected at the ventral male surface or the female tegument. The question of whether acetylcholine, available in the blood, might be a functional ligand for surface acetylcholinesterase and the AChR has been raised (Camacho *et al.*, 1996). An acetylcholine receptor was detected in one proteomic study of the tegument but proved to be of host origin (S. Braschi, personal communication).

2.2.3. *LDL Receptor*

The occurrence of a tegumental receptor for LDL in schistosomes was suggested since labeled LDL was shown to bind to schistosomula of *S. mansoni* (Rumjanek *et al.*, 1988; Chiang and Caulfield, 1989; Bennett and Caulfield, 1991; Xu and Caulfield, 1992). LDL binds to much of the parasite surface through charge interactions between cationic regions on the LDL and anionic-binding sites on overnight *in vitro* cultured schistosomula; much of the bound material can be displaced from the parasite surface by anions (Chiang and Caulfield,

1989). Purified human LDL bound to low molecular weight bands (~16–18 kDa) on blots of extracts of schistosomula that had been cultured overnight (Xu and Caulfield, 1992). PIPLC treatment removed the LDL-binding bands from schistosomula and resulted in the appearance of an LDL-binding band at ~17 kDa in the culture medium (Xu and Caulfield, 1992). These data suggest that the specific binding of human LDL is mediated by GPI-linked low molecular weight proteins. These proteins were not detected in adult extracts; instead, a 43-kDa protein is seen (Xu and Caulfield, 1992). Indeed, ¹²⁵I-labeled LDL was earlier reported to bind to a 43-kDa putative lipoprotein receptor (Sj43) in *S. japonicum* adult parasite extracts (Rogers *et al.*, 1989). However, in these experiments ¹²⁵I-labeled LDL did not bind to *S. mansoni* extracts (Rogers *et al.*, 1989). Similar ligand-binding assays suggested that Sj43 could bind apolipoprotein-B (apo-B), apo-E and human high-density lipoprotein (HDL), but not mouse HDL (Rogers *et al.*, 1989). Sj43 was localized at the parasite tegument and gut lining (Rogers *et al.*, 1989). The protein was purified using HPLC. Human LDL exhibited typical saturation kinetics on the purified protein with a calculated stoichiometry of 2 mol LDL/mol Sj43 (Rogers *et al.*, 1990). In more recent experiments, a human LDL fraction was reported to bind to adult schistosome tegumental proteins with different relative mobilities to those reported above; binding of human LDL to tegumental proteins with Mr of 60, 35 and 14 kDa was detected, using anti-human LDL antibody (Tempone *et al.*, 1997). Binding of LDL to tegumental membranes was also detected using microcalorimetry (Tempone *et al.*, 1997).

The presence of tegumental LDL receptors is suggested to be important for lipid capture by schistosomes, especially given their inability to synthesize lipids *de novo* (Meyer *et al.*, 1970; Tempone *et al.*, 1997). However, endocytosis of labeled LDL has never been reported. At 5 °C, labeled LDL binds to the outer tegument and the outer esophagus of schistosomula (Bennett and Caulfield, 1991). When worms are labeled at 5 °C and washed free of unbound material and then incubated at 37 °C, the label appears in the cecum (gut) of most of the worms after three hours. This result has led to the intriguing suggestion that the parasites may have the ability to sweep molecules bound to the surface into the gut as a potential mechanism

of nutrient acquisition or immune complex clearance (Bennett and Caulfield, 1991). In addition, since LDL binding at the surface can inhibit anti-schistosome antibody binding there, it was suggested that bound LDL can mask parasite antigens, possibly by simple steric hindrance (Chiang and Caulfield, 1989). The contribution of each of these numerous LDL receptor candidates to lipid binding by the parasites and whether they are exposed at the parasite surface is yet to be clarified. No clear LDL-receptor homolog has been identified in any analysis of the tegumental proteome (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

2.2.4. *Receptors for Proteins of the Complement Cascade*

Schistosomes have been reported to possess several proteins at their surface that are capable of binding to components of the host's complement cascade (Kabil, 1976; Santoro *et al.*, 1980; Ruppel *et al.*, 1984; Ruppel and McLaren, 1986; Rasmussen and Kemp, 1987; Silva *et al.*, 1993; Parizade *et al.*, 1994; Inal, 1999; Skelly, 2004). In this way, the parasites are proposed to inhibit the formation of membrane attack complexes that could damage or destroy the tegumental membranes. Evidence, sometimes conflicting, has been put forward for the presence of a C1-binding molecule, a C2-binding protein (CRIT), a C3-binding protein and a C8- and C9-binding protein (SCIP-1) at the surface of intravascular schistosomes. These proteins are described next.

(a) CRIT. A novel cDNA encoding a schistosome protein, now designated CRIT (for complement C2 receptor inhibiting trispannin), was identified from an adult *S. japonicum* cDNA library using serum obtained from vaccinated baboons (Inal, 1999). Vaccination was achieved by exposing the animals to irradiated cercariae. The cDNA clone possessed a 0.86 kb open reading frame potentially encoding a 286 amino acid protein of M_r 32 kDa. Anti-synthetic peptide antibodies detect the protein in the surface tegumental plasma membrane and tegumental surface pits of adult schistosomes by immuno electron microscopy. The *S. mansoni* homolog has been cloned and exhibits a similar localization pattern at the adult surface (Inal, 1999).

CRIT has recently been reported to act as a receptor for human complement protein C2. The extracellular domain of CRIT, preincubated *in vitro* with C2, is said to inhibit the classical pathway-mediated hemolysis of sheep red blood cells in a dose-dependent manner. Thus, CRIT has been suggested to act as a complement regulator by interfering with the formation of the C3 convertase, C4b2a (Inal and Sim, 2000; Inal and Schifferli, 2002). CRIT has 30% amino acid sequence identity with a human lysosomal-associated protein transmembrane 4 alpha (S. Verjovski-Almeida, personal communication). Its reported surface localization in schistosomes is therefore surprising, and warrants re-examination. It has not been detected in any of the tegument proteomic studies (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

(b) C3 receptor. Using specific anti-mouse C3 antisera, C3 was shown to be associated with the adult male tegument by immunohistochemistry but was only localized in tegumental infoldings and pits and not on its free surface (Rasmussen and Kemp, 1987). However, using anti-mouse C3 serum, earlier workers looked for the presence of C3 receptors on killed, whole parasites and detected C3 on the female tegument only and not on that of the male (Kabil, 1976). Older females (nine to 13 weeks) exhibited markedly greater fluorescence when compared to their younger (six week) counterparts (Kabil, 1976). Yet other studies, using immunofluorescence, reported no binding of C3 (or C1q or C4) to intact adult parasites (Linder and Huldt, 1983) or of C3 to schistosomula (Pearce *et al.*, 1990). Nonetheless, a 130-kDa protein was later reported to be a schistosome C3 receptor (Silva *et al.*, 1993). Labeled surface extracts contained a 130-kDa protein that bound to C3 sepharose and antibodies against a 130-kDa protein reveal this, in adult sections, in the surface membrane pits and in multilaminate vesicles within the tegument (Silva *et al.*, 1993). Metabolic labeling confirmed that the 130-kDa protein was synthesized by the parasite (Silva *et al.*, 1993). Given the controversy concerning C3 localization at the surface, co-precipitation studies are warranted to unambiguously confirm C3 binding to the parasite 130-kDa protein. The C3 receptor has been proposed to bind to C3 to inactivate it and limit complement-mediated damage (McGuinness and Kemp, 1981; Tarleton and Kemp, 1981). Others have reported

that C3 binding stimulates new plasma membrane formation and, in this way, the parasite negates any build-up of surface immune attack complexes (Zhou and Podesta, 1989).

Since the putative C3 receptor has not been cloned or sequenced, we cannot determine whether or not this molecule is listed in the published tegumental proteome (van Balkom *et al.*, 2005; Braschi *et al.*, 2006). However a C3 fragment has been identified at the tegument surface by biotinylation of live adult worms (Braschi and Wilson, 2006) (see Section 5.7). On the basis of molecular weight and peptide hits, it was identified as the C3c/C3dg fragment that results from the inactivation of C3 by complement regulatory proteins, after it has been fixed by C3 convertase (see Section 5.7).

2.3. The Many Faces of Paramyosin

Paramyosin is a 97-kDa myofibrillar glycoprotein expressed primarily in schistosome muscle cells that has been implicated by several studies as a potentially multi-functional host interactive molecule. The protein was identified as a key target of the humoral immune response in mice protectively immunized intradermally with soluble parasite extracts (Pearce *et al.*, 1986b). It is also a target of the human IgA response against *S. japonicum* (Hernandez *et al.*, 1999). Paramyosin has been proposed to interact directly with several complement molecules (notably C8 and C9, and possibly C1) as well as with immunoglobulin, to provide protection for the parasite against immunological attack. However, the localization of paramyosin outside of schistosome muscle tissue is controversial. The protein was shown to be present in the tegument and gut syncytium of adult parasites (Pearce *et al.*, 1986b; Lacleste *et al.*, 1995) and to be abundant in the outermost layer of the parasite, as determined by immuno EM (Matsumoto *et al.*, 1988). Clearly, if paramyosin is exposed in the outer tegument or is released from the parasites it could function to inhibit host complement components. However, later immunofluorescence analysis using *S. mansoni* adults reported that the protein is not detectable in the tegument (Schmidt *et al.*, 1996), and in *S. japonicum*, paramyosin was immunolocalized, using electron microscopy, to the surface of

lung-stage worms but not to the surface or tegument of the adult parasites (Gobert *et al.*, 1997). In a similar vein, some workers have been able to label paramyosin at the parasite surface (Loukas *et al.*, 2001), while others could not (Davies and Pearce, 1995; Skelly and Shoemaker, 1996; Braschi and Wilson, 2006). Finally, paramyosin has not been detected following proteomic analysis of tegument surface membranes (van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

2.3.1. *Schistosome Complement Inhibitory Protein-1 (SCIP-1/paramyosin)*

Antibodies against the human complement inhibitor protein CD59 detect an ~94-kDa protein in schistosomula and adult worm extracts (Parizade *et al.*, 1994). These antibodies also label the surface of the parasites as detected by immunofluorescence and immuno-EM analysis (Parizade *et al.*, 1994). The protein, originally designated SCIP-1 (schistosome complement inhibitory protein-1), can be released from 24-hour-old schistosomula following treatment with PIPLC, indicating that it is linked to the external membrane through a GPI anchor (Parizade *et al.*, 1994). Soluble SCIP-1, partially purified from schistosome tegument extracts using anti-CD59 antisera, binds to purified human C8 and C9 and inhibits the lysis of sheep and rabbit red blood cells by human complement (Parizade *et al.*, 1994). Sequence analysis of purified SCIP-1 revealed it to be paramyosin (Deng *et al.*, 2003). Native and recombinant paramyosin was shown to bind human C8 and C9 and to inhibit C9 polymerization onto red blood cells (Deng *et al.*, 2003). Like the human protein CD59, SCIP-1 (paramyosin) is proposed to prevent complement activation at the terminus of the cascade.

2.3.2. *C1 Receptor/Paramyosin*

The complement component C1q was fluorescein-labeled and shown to bind to three-hour-old schistosomula (Santoro *et al.*, 1980). No binding was observed to cercariae, 24-hour-old schistosomula, lung worms or adult parasites (Santoro *et al.*, 1980). Pretreating the three-hour

schistosomula with IgG resulted in a substantial increase in subsequent C1q binding to these parasites (Santoro *et al.*, 1980). The data were interpreted to mean that C1q could bind to the parasites by both a specific receptor and by IgG attached to the parasite surface. While no specific C1q receptor has been isolated from the schistosome tegument, paramyosin has been shown to bind complement component C1 *in vitro* and inhibit the classical complement pathway (Laclette *et al.*, 1992; Schmidt *et al.*, 1996).

2.3.3. *Fc Receptor/Paramyosin*

Because of the reported ability of adult schistosomes to adsorb heterospecific antibody onto their tegumental surfaces via their Fc domains (see Section 5.8), attempts were made to identify this Fc receptor. Adult parasites were first surface biotinylated and extracts were then incubated with human IgG-Fc coupled to sepharose. A 97-kDa biotinylated protein bound to the sepharose complex and was identified as paramyosin (Loukas *et al.*, 2001). Paramyosin-IgG complexes were detected on the surface of freshly perfused adult *S. mansoni*, using protein G-sepharose (Loukas *et al.*, 2001). In contrast, in experiments by other workers mentioned earlier, paramyosin was not available for surface biotinylation (Davies and Pearce, 1995; Skelly and Shoemaker, 1996; Braschi and Wilson, 2006).

The ability of paramyosin to bind such a diversity of host proteins must raise questions about the specificity of these interactions, irrespective of whether it is localized on the tegument surface. Indeed the range of proteins bound could be used to argue that isolated paramyosin exhibits a degree of natural 'stickiness'. This conclusion is reinforced by the failure to detect paramyosin in proteomic analyses of the tegument surface membranes (Braschi and Wilson, 2006; Braschi *et al.*, 2006).

2.4. Myosin Heavy Chain

As noted earlier, mice that have been exposed to radiation-attenuated *S. mansoni* cercariae are highly resistant to challenge infection.

Among the antigens recognized by sera from these protected mice is a 200-kDa glycoprotein (Soisson *et al.*, 1992). A cDNA clone encoding a 62-kDa fragment of this antigen was identified using polyclonal antiserum raised against a schistosome glycoprotein fraction that showed greater or unique immunogenicity in resistant versus chronically infected mice (Dalton and Strand, 1987; Soisson *et al.*, 1992). This cDNA clone, designated irradiated vaccine-5 (IrV-5), encodes part of schistosome myosin β -heavy chain (that also possesses sequence similarity to *S. mansoni* paramyosin of 40%) (Soisson *et al.*, 1992). Mice vaccinated with recombinant IrV-5 (rIrV-5) in various formulations exhibited 15–83% protection from challenge infection (Soisson *et al.*, 1992). Sera from these mice, as well as an mAb generated against purified rIrV-5, bound to the surface of schistosomula as determined by immunofluorescence (Soisson *et al.*, 1992). As was the case for paramyosin mentioned above (Section 2.3), the surface localization of myosin outside of muscle is similarly controversial. For instance, the *S. japonicum* IrV-5 homolog (Sj62) could not be detected at the parasite surface (but was seen in the subtegumental muscle layers in adult worm sections) (Zhang *et al.*, 1998). In addition, while vaccination of several groups of mice, rats or pigs with recombinant Sj62 induced high titers of specific anti-Sj62 antiserum, in only one experimental group of mice was there a significant reduction in worm burden (27%) (Zhang *et al.*, 1998; Shi *et al.*, 2001). Myosin heavy chain is not detected by proteomic analysis of adult tegumental fractions (Braschi and Wilson, 2006; van Balkom *et al.*, 2005; Braschi *et al.*, 2006).

2.5. Nutrient Transporters

2.5.1. Schistosome Glucose Transporter Proteins

Schistosomes in the bloodstream transport glucose and other nutrients across their outer tegument (Fripp, 1967c; Skelly *et al.*, 1998). This implies the presence of host exposed glucose transporter proteins. A family of three different glucose transporters from *S. mansoni* (designated SGTP1, SGTP2 and SGTP4) have been cloned and

characterized (Zhong *et al.*, 1995; Jiang *et al.*, 1996; Skelly and Shoemaker, 1996, 2000, 2001). Using the *Xenopus* expression system it has been shown that SGTP1 and SGTP4 are functional facilitated diffusion glucose transporters; SGTP2 is not functional in this assay.

SGTP4 is localized uniquely and specifically to the tegument of adult males and females. Using confocal microscopy and immuno EM, tegumental localization for SGTP1 and SGTP4 was shown to be distinctly asymmetric. SGTP1 was found only in the basal membranes, and SGTP4 only in the apical membranes and tegumental secretory bodies (Zhong *et al.*, 1995; Jiang *et al.*, 1996). SGTP4 (but not SGTP1) could be readily labeled by aqueous biotinylating agents on the surface of living worms demonstrating that it is present at the host/parasite interface. SGTP4 exhibits stereo specificity for glucose. Like other members of the glucose facilitated diffusion family, SGTP4 exhibits sodium independence and is markedly inhibited by phloretin and cytochalasin B. In oocytes, the K_m of SGTP4 for 3-*O*-methylglucose is 2 mM (Skelly and Shoemaker, 1996).

In recent proteomic analysis of the tegument, SGTP4 has been detected in the mass spectrometric analysis of a tegument-enriched extract in one study (van Balkom *et al.*, 2005) but not in a second study (Braschi *et al.*, 2006) which did find SGTP1. However, in later work, both glucose transporters were detected by LC-MS analysis of highly enriched surface membranes (S. Braschi, personal communication).

Since SGTP4 is found only in the mammalian-stage tegumental membranes, the timing and nature of its appearance on the worm surface following invasion of the vertebrate host was investigated. SGTP4 is detected first within the body of the invading parasite in a network of interconnected cytons. As the parasite transforms into the intra-mammalian stage, the protein is then detected in the cytoplasmic connections to the surface, and next it is deposited at the surface in discrete patches (Skelly and Shoemaker, 2000, 2001). Over the following several hours, as the patches coalesce, the protein is finally deposited in a contiguous layer over most of the worm surface. These results support the basic model for the origin of the schistosomulum, tegument surface proposed by Hockley and McLaren, based on electron microscopical observations of parasite sections (Hockley and McLaren, 1973).

2.5.2. *Amino Acid Permeases*

Experiments on amino acid uptake by adult male schistosomes *in vitro* have suggested the presence of at least five distinct amino acid importing systems (Asch and Read, 1975). A protein has been identified in *S. mansoni* that likely represents the molecular basis of one of the five amino acid uptake systems described in whole worms (Skelly *et al.*, 1999). This ~55-kDa protein has been designated SPRM1lc (for Schistosome Permease 1 Light Chain) and belongs to the heterodimeric amino acid permease protein family (Skelly *et al.*, 1999). When expressed in *Xenopus* oocytes, these proteins interact with a type II glycoprotein (the heavy chain) to reveal their amino acid transport capabilities (Mastroberardino *et al.*, 1998). While the schistosome heavy chain partner for SPRM1lc has not yet been identified, SPRM1lc does exist as part of a high molecular weight complex that can be disrupted by reducing agents. This is consistent with the notion that some of the SPRM1lc in schistosomes is linked by a disulphide bridge to its heavy chain partner. When expressed in *Xenopus* oocytes together with a heterologous heavy chain, SPRM1lc is capable of transporting several amino acids, notably the basic amino acids, histidine, arginine and lysine. In addition, transport of leucine, phenylalanine, methionine and glutamine is enhanced in this system (Skelly *et al.*, 1999).

SPRM1lc is detected in all schistosome life stages examined (eggs, sporocysts, cercariae and adult males and females) (Skelly *et al.*, 1999). In adult parasites, the protein is widely distributed throughout the parenchyma as well as in the tegument and tegumental cytons (Skelly *et al.*, 1999). Laser scanning confocal microscopy reveals that SPRM1lc is located in the outer tegument, where it likely functions to import amino acids across the surface of the tegument from the host bloodstream. The wide distribution of SPRM1lc within the adult worm suggests that the protein is also important in distributing amino acids throughout the organism. While SPRM1lc is not detected in the tegumental proteome by MS, another amino acid transporter with homology to the B amino acid transporter (BAT) family has been noted (van Balkom *et al.*, 2005; Braschi *et al.*, 2006). Indeed it is possible that SPRM1lc and the BAT protein form a heterodimeric pair to facilitate amino acid uptake at the tegument surface.

2.6. Structural Proteins and Those of Unknown Function

2.6.1. *Sm23*

The 23-kDa parasite membrane protein, Sm23, was originally identified as the target of a monoclonal antibody that had been prepared using mice immunized with a surface membrane-enriched schistosomula preparation (Harn *et al.*, 1985). Later, this antigen was isolated by immunoscreening an adult worm cDNA library with antibody affinity purified on the 23–25-kDa integral membrane protein parasite fraction (Wright *et al.*, 1990). The predicted protein is 218 amino acids long and contains four putative transmembrane regions (Wright *et al.*, 1990). The protein also contains a GPI anchor (Koster and Strand, 1994b). Sm23 and its homolog from *S. japonicum* (Sj23) have been found to be widely distributed in several tissues and to immunolocalize to the surface of newly transformed and developing schistosomula (Harn *et al.*, 1985; Wright *et al.*, 1991). Sm23 is detected in cercariae, schistosomula, lung worms and adults (Harn *et al.*, 1985). The protein has been shown to be associated with caveolae-like structures in the adult parasite surface membrane (Racoosin *et al.*, 1999).

Sm23 exhibits 30–36% similarity to several human hematopoietic or tumor-associated antigens (Wright *et al.*, 1990). In spite of this low level of sequence similarity, Sm23 exhibits strong ‘domain homology’ with proteins of the ‘tetraspanin’ family (Maecker *et al.*, 1997). Tetraspanins have been proposed to play a role in cell adhesion, migration and survival; antibody binding to these proteins can trigger changes in phosphorylation patterns within cells (Maecker *et al.*, 1997; and references therein). This has led to the suggestion that members in this protein family are involved in signal transduction. Sm23 is detected in the surface membranes in one study of the tegumental proteome (Braschi *et al.*, 2006) but not in the other (van Balkom *et al.*, 2005). Two other members of the tetraspanin protein family have also been detected in the tegumental membranes by MS analysis (Braschi *et al.*, 2006).

Sm23 is among the antigens recognized by sera from mice multiply vaccinated with radiation-attenuated cercariae (Richter *et al.*, 1993b). Such sera can passively transfer resistance against cercarial challenge to naive mice (Richter and Harn, 1993; Richter *et al.*, 1993a). Sm23 is

immunogenic in infected humans as well as in rabbits exposed to *S. mansoni* (Wright *et al.*, 1990; Koster *et al.*, 1993). Its B and T cell epitopes have been mapped (Reynolds *et al.*, 1992), and the gene cloned and characterized (Lee *et al.*, 1995).

Immunization of experimental animals with Sm23 has been shown to induce a protective immune response in several vaccine trials (Da'dara *et al.*, 2001b, 2002). Antibody isotyping suggests that greatest protection is associated with a Th1 type immune response directed against the protein (Da'dara *et al.*, 2001b, 2002). Immunization of animals with Sj23 have given mixed results: mice immunized with plasmid DNA encoding this molecule produced anti-Sj23 IgG antibodies, but were not protected from challenge infection with *S. japonicum* cercariae (Waine *et al.*, 1999). In contrast, two of the three laboratory and field vaccination trials with Sj23 in sheep and water buffalo induced significant protection (~40%) (Shi *et al.*, 2001).

2.6.2. A1.12/9

The A1.12/9 protein family is a group of several antigens found in *S. mansoni* adults (Havercroft and Smith, 1993). In lung-stage and older parasites, A1.12/9 can be detected in the tegumental matrix and associated with the outer tegumental membrane (Havercroft and Smith, 1993). In addition, A1.12/9 can be detected in neurons and in the putative sensory receptors of cercariae as well as intra-mammalian-stage parasites (Havercroft and Smith, 1993). Some homology with mammalian chromogranin A has led to the suggestion that A1.12/9 belongs to the granin protein family and, by analogy, may modulate host parasite interactions by interfering with host protein secretion (Havercroft and Smith, 1993). However, molecules of this nature have not been detected in recent proteomic analysis of the tegument (Braschi and Wilson, 2006; Braschi *et al.*, 2006).

2.6.3. Spine Glycoprotein

Monoclonal antibodies were prepared against a partially purified fraction of cercarial glycoproteins. One monoclonal antibody extensively

stained the surface of three-hour schistosomula and adult worms after fixation in formalin/PBS; the spines attached to the tubercles on the male dorsal surface were 'brightly illuminated' (Norden *et al.*, 1982). Spines on the female worms were similarly stained. Using the anti-body, a single protein of 170 kDa was identified in cercariae and was shown to be recognized by sera from infected humans (Norden *et al.*, 1982). A 170-kDa glycoprotein was purified from adult extracts using the monoclonal antibody. Note that since spines are intracellular, it remains unclear whether the spine glycoprotein is actually in the surface membranes. In addition, and as stated earlier, spines protruding from tubercles may be abraded most easily during specimen preparation to expose intracellular molecules.

2.6.4. 200-kDa Protein

A 200-kDa (Sm200) protein is reported to be detected on the surface of adult worms by MAbs prepared from spleen cells of mice exposed to radiation-attenuated cercariae as well as by MAbs prepared from spleen cells of mice infected with only male schistosomes (Sauma and Strand, 1990). The 200-kDa protein has been shown to incorporate [³H]myristic acid (Sauma *et al.*, 1991). It can be released from intact adult parasites following phospholipase C (PIPLC) treatment suggesting its exposed surface location (Sauma and Strand, 1990; Sauma *et al.*, 1991). cDNA clones encoding the protein were isolated and a GPI anchoring signal was identified in its predicted sequence (Hall *et al.*, 1995). The 200-kDa protein is novel and has no similarity to other sequences in the databases (Hall *et al.*, 1995). Sm200 was not detected by MS analysis of tegument preparations (van Balkom *et al.*, 2005; Braschi *et al.*, 2006) but was accessible to biotinylation in live worms (Braschi and Wilson, 2006). Its release by PIPLC treatment of live adult worms has been confirmed by tandem MS (Borges, unpublished data).

2.6.5. Sm25

Some proteins, whose localization at the host-parasite interface remains controversial, deserve mention. For instance, an Mr 25 000

protein (Sm25) is a major target of antibody following vaccination of mice with an adult tegumental membrane extract (Smithers *et al.*, 1989). The titer of anti-Sm25 antibody generated in the mice correlates with protection (Smithers *et al.*, 1989). Sm25 is an integral membrane glycoprotein whose cDNA was cloned (Ali *et al.*, 1991). The protein is palmitoylated via a thioester bond which has been suggested to stabilize it in the membrane (Pearce *et al.*, 1991). The accessibility of Sm25 to exogenous protease led to the suggestion that the protein is exposed at the host-parasite interface (Pearce *et al.*, 1991). However an inability to label Sm25 with biotin on parasites suggested that the protein is located predominantly beneath the surface (Suri *et al.*, 1997). Ultrastructural localization of Sm25 by EM confirms that Sm25 is located in the tegument, but is not associated with the outer membrane (Abath *et al.*, 1999). This may explain the inability of a polypeptide fragment of Sm25 to provide protection in vaccination trials (Suri *et al.*, 1997). However, it has been identified by LC-MS in several fractions derived from a highly enriched tegument membrane preparation (S. Braschi, personal communication).

2.7. Additional Tegumental Proteins Identified by Proteomics

Other proteins likely present in the outer tegumental membranes include those involved in ion balancing. Indeed, a CaATPase homolog has been immunolocalized to the adult tegument (Da'dara *et al.*, 2001a) and Ca-binding proteins have been identified in apical tegumental membrane preparations (Siddiqui *et al.*, 1991). Evidence exists for the presence of a NaKATPase in the tegument, and a NaKATPase α -chain has been characterized in schistosomes (SNaK1) (Skelly *et al.*, 2001). In contrast to immunolocalization data, which places SNaK1 mostly to the peripheral muscle band and not the tegument (Skelly *et al.*, 2001), proteomic data suggest that this membrane protein is in the tegument (Braschi and Wilson, 2006; Braschi *et al.*, 2006). Indeed a NaKATPase β -chain homolog has also been detected by proteomics in this location suggesting that functional sodium/potassium exchange occurs at the host-parasite interface.

Table 2 Schistosome membrane and membrane-associated proteins obtained from proteomic analysis of isolated and enriched tegument surface membranes. Ref #1, Braschi *et al.*, 2006; Ref #2, Braschi and Wilson, 2006; Ref #3, Braschi and Wilson, 2006. Ref #3 yields a large number of protein hits; this work involved tegument differential extraction followed by fraction analysis by LC MS from a very large quantity of adult parasites. ‘Long’ and ‘short’ refer to the use of long- and short-form biotinylation reagents. Schistosome accession numbers are from version 1 of the genome database at www.SchistoDB.org. Sm numbers refer to assembled contigs. Phat, snap and GlimmerHMM to gene finding predictions

Accession number	Protein name	Ref #1	Ref #2		Ref #3
			Long	Short	
Transporter proteins					
Sm11978	Schistosome glucose transporter protein 1 (SGTP1)	x			x
snap43116	Schistosome glucose transporter protein 4 (SGTP4)				x
Sm01225	Amino acid transporter SLC3A1 (rBAT)	x			x
Sm07319	Na + /K + transporting ATPase (SNaK1) alpha subunit	x		x	x
Sm00144	Na + /K + transporting ATPase beta 1a	x			x
Phat02905	Plasma membrane calcium-transporting ATPase 2	x			x
Sm02430	Plasma membrane calcium-transporting ATPase 3				x
Phat08744	Plasma membrane calcium ATPase 3 isoform 3b				x
Sm27215	Cation-transporting ATPase 3				x
Sm03357	Copper transporter	x			\
snap28998	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4				\
Phat05022	Phospholipid-transporting ATPase IA				x
snap28221	Phospholipid-transporting ATPase IG				x
Sm19989	Phospholipid-transporting ATPase IIB				x

Table 2 (Continued)

Accession number	Protein name	Ref #1	Ref #2		Ref #3
			Long	Short	
Sm00376	Aminophospholipid transporter ATPase, type 8A, member 1				x
Sm03693	Aquaporin	x			x
Membrane structural proteins					
Sm12172	Tetraspanin A	x			x
Sm02886	Tetraspanin B (Sm23)	x			x
Sm04463	Tetraspanin C (TE736)	x		x	x
Sm12366	Tetraspanin D (CD63-like protein Sm-TSP-2)		x	x	x
Sm02775	Dysferlin	x	x	x	x
Sm00204	Annexin 11a, isoform 2				x
Sm03987	Annexin VI	x	x	x	x
Sm12950	Sorcin	x			x
Sm12654	Phospholipid scramblase 1				x
Sm12683	Syndecan binding protein (Syntenin)				x
Membrane enzymes					
Sm12745	ATP-diphosphohydrolase	x	x	x	x
snap05486	Phosphodiesterase	x	x	x	x
Sm00962	Alkaline phosphatase	x	x	x	x
snap25325	Calpain-B	x		x	x
snap16831	Acetylcholinesterase				x
Membrane other					
Sm04760	Gp18–22 (Sm25)				x
snap04278	Multidrug resistance protein 2				x
Sm03865	200-kDa surface protein (Sm200)			x	x
snap20471	Fibronectin type III				\
glimmer04896	DeltaB				\
C608019.1	Tyrosinase	x			
Secreted proteins					
Sm09193	Sm29	x	x	x	x
Sm12315	Sm13				x
Unknowns					
Sm00749				x	x
Sm01352		x			x
Sm03716		x			x
Sm05431		x			x
Sm07392	Tetraspanin-like		x	x	x
Sm09712					x
Sm11042		x			x

(Continued)

Table 2 (Continued)

Accession number	Protein name	Ref #1	Ref #2		Ref #3
			Long	Short	
Sm11921	Tetraspanin-like		x	x	x
Sm12172		x			x
Sm13027		x			x
Sm13096				x	x

Proteomic analysis of the adult outer tegumental membranes by mass spectroscopy has provided direct data on their composition (Table 2). The information obtained is only as good as the quality of the surface preparations obtained, but where the same molecules are revealed by the different approaches, we can be reasonably confident about their presence in, or association with the plasma membrane and membranocalyx. Conversely, the failure to find proteins by tandem mass spectrometry, which have been reported at the surface, does not rule them out. However, as the body of information grows, it will become apparent that some putative tegument surface proteins are not located there.

Table 2 lists several transporter proteins at the surface: the potential for uptake of sugars and amino acids at the surface is confirmed, together with inorganic ions such as Na, K, Ca and Cu. The presence of an aquaporin also suggests water flux across the surface. Only two proteins appear to fall into the secretory category (Sm29 and Sm13). Membrane structural proteins are prominent, perhaps related to the complex configuration of the tegument surface. These include four distinct tetraspanins and two annexins. It has been suggested that the latter might conceivably serve to anchor the membranocalyx to the plasma membrane, due to their phospholipids binding properties. The five enzymes detected have all been previously reported at the tegument surface by a variety of techniques. However, it is notable that there is no evidence for receptors capable of signaling to the nucleus in the subtegumental cytons. This could be a matter of sensitivity of proteomic techniques, but it is possible that the membranocalyx external to the plasma membrane precludes detection of signaling macromolecules in host plasma.

2.8. GPI-Anchored Surface Proteins

Several of the tegumental proteins detailed in this report have been described as being surface-associated through GPI anchors since PIPLC treatment of intact parasites can release the molecules. These include m28 (elastase), acetylcholinesterase, alkaline phosphatase and SCIP-1 (paramyosin). Sm23, Sm200 and the 16–18 kDa reported LDL-binding proteins of schistosomula (Sauma *et al.*, 1991; Xu and Caulfield, 1992; Parizade *et al.*, 1994). In several cases, cDNAs encoding these proteins have been isolated and the proteins do not contain predicted GPI anchoring sites (for instance, AChE, Sm23, SCIP-1 (paramyosin)). Some of the reported GPI-anchored proteins (for instance, alkaline phosphatase, Sm200 and Sm23) have been identified in caveolae-like structures in the adult surface membrane (Racoosin *et al.*, 1999), in agreement with work in other organisms showing the presence of GPI-anchored proteins in specialized micro domains (Ferguson, 1994).

GPI anchors facilitate increased lateral and rotational mobility within the plane of the membrane. GPI anchors can also facilitate the release of surface proteins through the action of phospholipases. GPI-anchored proteins often predominate on cell surfaces facing the outside of an organism. One potential advantage of GPI-anchored proteins versus those that possess transmembrane domains may be the physical insulation of cell surface molecules that have exclusively extracellular functions from the cytoplasm (Ferguson, 1994). However, GPI-anchored proteins can be involved in generating secondary messengers within cells, to activate a variety of cellular functions. Note that some schistosome proteins are reported to be expressed in transmembrane form and GPI-anchored form, e.g. Sm23 (Koster and Strand, 1994b).

3. TEGUMENTAL LIPID COMPOSITION

Adult schistosome tegumental membrane extracts were found to contain high concentrations of phospholipids and cholesterol, when analyzed by thin layer chromatography (Rogers and McLaren, 1987). The phospholipid composition of surface membrane extracts from

six-week adult parasites showed a typical plasma membrane profile with a high sphingomyelin content ($\sim 20\%$) (Allan *et al.*, 1987). Younger forms (two to three-weeks old) had negligible amounts of sphingomyelin (Rogers and McLaren, 1987). The phospholipids, phosphatidylcholine and phosphatidyl ethanolamine, are major constituents of the surface membranes. More phosphatidylcholine than phosphatidyl ethanolamine can be found in the membranes of two- or three-week juvenile-stage worms, whereas the reverse is true of membranes prepared from mature adult parasites (Rogers and McLaren, 1987). Certainly, phosphatidyl ethanolamine is the major phospholipid to be surface labeled (Roberts *et al.*, 1983). Despite this, in a multilaminate vesicle-enriched fraction from adult worms, phosphatidylcholine was far more abundant than phosphatidylethanolamine (57% versus 17%) (McDiarmid *et al.*, 1982). Recall that multilaminate vesicles are proposed to be the precursors of the outer tegumental membranes.

Lysophosphatidylcholine was also detected in a multilaminate vesicle fraction purified from adult worm homogenates by sucrose gradient centrifugation (McDiarmid *et al.*, 1982). Lysophosphatidylcholine, as well as phosphatidylethanolamine, has been implicated in membrane fusion and may facilitate the merger of multilaminate vesicles with the surface plasma membrane (McDiarmid *et al.*, 1982) or may facilitate the fusion of the surface membrane with host cell membranes (see Section 5.9). Sphingomyelin is detected at $\sim 9\%$ of the total phospholipid. Additionally, a high level of activity of phosphatidic acid phosphatase is reported in the multilaminate vesicle fraction suggesting that these vesicles are engaged in the modulation of the surface material (McDiarmid *et al.*, 1982). Phosphatidic acid phosphatase can hydrolyze phosphatidic acid to form diacylglycerol—a phospholipid precursor.

The six-week adult tegumental membranes contained at least five glycolipids, four of which were highly polar (Rogers and McLaren, 1987). The fatty acid composition of surface phospholipids was remarkable because of the presence of an unusual unsaturated fatty acid (C20:1) identified as eicosaenoic acid (Rogers and McLaren, 1987). Since this fatty acid is found in extremely low amounts in mammalian membranes, schistosomes (while lacking the capacity to synthesize fatty acids *de novo*) must have the capacity to generate eicosaenoic acid.

4. TEGUMENTAL CARBOHYDRATE COMPOSITION

Thirty years ago, the presence of carbohydrate at the schistosome surface was demonstrated by several groups, who examined the ability of lectins such as concanavalin A (Con A) and wheat germ agglutinin (WGA) to bind to the parasites (Bennett and Seed, 1977; Murrell *et al.*, 1978; Torpier and Capron, 1980). These studies indicated the presence of either glycoproteins and/or glycolipids containing complex oligosaccharides on the parasite's membranocalyx (Simpson and Smithers, 1980). However, the ability of schistosomes to acquire host glycolipids on the tegument surface (see Section 5), readily demonstrated by immunocytochemistry, complicates the analysis of glycan constituents. It is not possible at present to say whether the glycans revealed are of host origin, parasite origin or a mixture of both. However, it seems certain that at least some of the surface proteins are glycosylated. Furthermore, evidence that components of the membranocalyx are glycosylated is provided by the observation that when adult worms are incubated for 30 minutes with Con A, multilaminate vesicles are released into the surface pits but fail to unfold, presumably due to cross-linking of their glycans; this clearly indicates the presence of endogenous glycan in the membranocalyx (Wilson and Barnes, 1977). Carbohydrate staining can also be detected in multilaminate vesicle contents at the ultrastructural level, although much fainter than in discoid bodies (Wilson and Barnes, 1974b).

In one study of lectin binding, trypsin and pronase treatment of live parasites failed to remove Con A and WGA-binding sites (Murrell *et al.*, 1978). This led to the suggestion that the lectin receptors are either inaccessible to these enzymes or are incorporated into polysaccharides or glycolipids rather than glycoproteins (Murrell *et al.*, 1978). However, tegumental surface extracts, resolved by SDS-PAGE, do contain many Con A-binding glycoproteins (Simpson *et al.*, 1983a). Tegumental extracts (prepared by freeze thawing the parasites or by treating them with saponin) resolve into more than 20 bands by SDS-PAGE. Most bands stain positively for the presence of carbohydrate, suggesting that the major proteins of the tegument are glycosylated (Cordeiro and Gazzinelli, 1979).

An adult worm tegumental tissue extract was analyzed for its glycosaminoglycan (GAG) content revealing the presence of heparin and/or heparin sulfate and chondroitin sulfate (Robertson and Cain, 1985). The vast majority of the parasite's GAGs was in the tegumental fraction (~73%) based on uronic acid content and this has been suggested to reflect the parasite's possible use of GAGs, like heparin, as anticoagulants at the surface (Robertson and Cain, 1985).

Schmidt reported intense binding to the adult schistosome surface of a number of gold-labeled lectins (from *Erythrina cristagalli*, ECL; *Datura stramonium*, DSL; *Triticum vulgaris*, wheat germ, WGA; *Ricinus communis*, RCA 1; and *Glycine max*, SBA) as determined by electron microscopy (Schmidt, 1995). Weaker binding of Con A, peanut agglutinin (PNA) and *Wisteria floribunda* lectin (WFL), and no detectable binding of AAL (*Anguilla anguilla* lectin), UEA 1 (*Ulex europaeus* agglutinin), LTL (*Tetragonolobus purpureas* lectin) and DBA (*Dolichos biflorus* agglutinin) was reported. This work concluded that both male and female parasites are entirely and evenly covered with glycans and that this covering was only seen when the parasites were briefly fixed with glutaraldehyde. Electron microscopy revealed that the surface glycans appeared as an amorphous mucus of low electron density (Schmidt, 1995). In earlier work, fixation of adult flukes with ruthenium red revealed the presence of a negatively charged layer external to the parasite's outer plasma membrane and suggested the presence of a carbohydrate-rich 'glycocalyx'. The adult surface exhibited an array of dense aggregated material (McDiarmid and Podesta, 1984). The precise biochemical nature of the glycans that compose this covering at the surface is unclear: lectin-binding studies show large amounts of exposed *N*-acetylglucosamine (i.e. galactose (β 1-4) *N*-acetylglucosamine or Gal β 1-4GlcNac) (Schmidt, 1995). It was suggested that these parasite surface molecules, being common components of vertebrate glycans, are not immunogenic and may therefore mask the parasites against immunological attack (Schmidt, 1995). Indeed, as noted, the possibility that some or all of these carbohydrates are actually derived from the vertebrate host must be considered (see Sections 5.1 and 5.2).

The presence of sialic acid at the schistosome surface was suggested by treating parasites with the sialic acid cleavage enzyme

neuraminidase to expose new lectin (soybean agglutinin)-binding sites (Simpson and Smithers, 1980). In addition, neuraminidase treatment removed much of the surface coat material previously revealed by rubidium red fixation (McDiarmid and Podesta, 1984). However, no parasitic helminth appears to synthesize sialic acid-containing glycans suggesting a lack of the many genes required for the synthesis and mobilization of sialic acid (Nyame *et al.*, 2004), nor has transialidase activity been reported. This makes it likely that any sialic acid residues reported at the schistosome surface are host-derived.

The fucose-specific lectins (UEA I, LTL, AAL) and fucose-binding protein were scarcely or were not bound to the parasite tegument (Simpson and Smithers, 1980; Schmidt, 1995), suggesting the absence of exposed fucose residues. If we assume that much of the parasite surface carbohydrate is derived from the mammalian host and given that such host glycans are low in fucose, perhaps this result is not surprising. However, some fucosylated *N*-acetylglucosamines have been reported at the schistosome surface; the fucose-containing trisaccharide Gal β 1-4(Fuc α 1-3)GlcNAc β 1 (designated Lewis^x (Le^x) or CD15) has been located very widely at the surface of intra-mammalian-stage schistosomes using anti-Le^x MAbs (Koster and Strand, 1994a; Remoortere *et al.*, 2000). Surface expression of the Le^x epitope was reported to be initiated after the transformation of cercariae to schistosomula and was maintained throughout the adult life in both sexes (Koster and Strand, 1994a). Le^x is also detected in the adult gut and on material released by the parasites (Koster and Strand, 1994a; Remoortere *et al.*, 2000). Le^x elicits an immune response in infected hosts (Koster and Strand, 1994a; Nyame *et al.*, 1996; Remoortere *et al.*, 2000; van Remoortere *et al.*, 2001). An MAb against Le^x directs effector eosinophils to kill schistosomula *in vitro* (Ko *et al.*, 1990). Since the localization results were obtained using adult parasite sections (Remoortere *et al.*, 2000) or fixed adults treated with detergent (NP-40) (Koster and Strand, 1994a), whether the Le^x moiety is actually exposed *in vivo* remains unclear.

Some selectins (carbohydrate-binding glycoproteins involved in adhesion and inflammation) recognize fucosylated ligands, including Le^x. Le^x is a weak ligand for P-selectin. Is Le^x, therefore, important for schistosomes in sensing their environment? Could such ligands

play a role in parasite migration—their Le^x binding to selectins in specific anatomical sites to provide geographical information to the parasite?

Le^x is present on the α and β chain of lymphocyte functional antigen-1 (LFA-1) which is a ligand molecule for intercellular adhesion molecule-1 (ICAM-1). It is conceivable that parasite-derived Le^x could interfere with lymphocyte binding to ICAM-1 on, e.g. antigen presenting cells (APCs) and/or that anti- Le^x Abs could impede LFA binding in selectin-dependent leukocyte adhesion. ICAM-1 can provide a signal for T cell activation (Chirathaworn *et al.*, 2002). It is also possible that parasite Le^x could interfere with ICAM-1 binding to T cells to prevent such activation. Such effects could impede host immunological function and promote parasite survival. In support of this notion, *in vitro* killing of schistosomula via antibody-dependent macrophage-mediated cytotoxicity has been shown to be inhibited by free monomeric Le^x or by monoclonal anti- Le^x antibody (Trottein *et al.*, 1997). Alternatively, Le^x production coupled to the production of anti- Le^x immunity may dampen the impact of each and prevent either from exerting a substantive immunomodulatory effect *in vivo*.

Studies with antibodies to sialyl Le^x suggest that fresh schistosomula display a surface lectin with selectin-like properties that may be involved in recognition of sialyl Le^x on, e.g. host leukocytes (Trottein *et al.*, 1997). Such a schistosome surface molecule, like Le^x described above, could also play a role in parasite-induced immunomodulation and/or parasite environmental sensing and migration. However, as pointed out earlier, with all studies involving fresh schistosomula, there is a proposed transient exposure of internal antigens onto the surface during and immediately following cercarial transformation and this may complicate any interpretation of surface studies obtained using this life stage.

5. HOST MOLECULES

A variety of host molecules have been demonstrated, or implicated, at the surface of the intra-mammalian-stage schistosome. They belong

Table 3 Host molecules reported in the literature as being at the host parasite interface. Ref #1, Braschi *et al.*, 2006; Ref #2, Braschi and Wilson, 2006

Molecule	Description	Evidence for surface location	Life stage examined	Adult tegument proteome analysis	
				Ref #1	Ref #2
Blood group antigens	Glycan antigens (e.g. A, B and Lewis ^b *) detected; protein antigens (e.g. M.N, Duffy) not detected	IF, agglutination, worm transfer experiments	Somula, adult	Carbohydrate	
MHC glycoproteins	Class I and class II alloantigens	IF	Lung worm, adult	No	No
Immunoglobulin	Conflicting immunoglobulin binding data	IF	Somula, adult	No	Yes
Delay accelerating factor (DAF)	70-kDa complement inhibitory molecule	IF, surface labeling	Somula, adult	No	No
Skin antigens	Uncharacterized	IF	Somula	-	-
Fibronectin	Whether of host or parasite origin is unresolved	IF, immunohistochemistry	Somula, adult	No	No
α2-Macroglobulin	Whether of host or parasite origin is unresolved	Immuno-EM, agglutination	Adult	No	No

to several classes and include LDL (see Section 2.3 on LDL receptor analysis), blood group antigens, MHC molecules, fibronectin, immunoglobulin, α2-macroglobulin, delay accelerating factor (DAF) and skin antigens. Table 3 lists host molecules described in the literature at the schistosome surface, while Table 4 focuses on host molecules identified at the surface by proteomics.

5.1. ‘Host Antigens’

The presence of antigenic epitopes shared between schistosomes and their hosts was first suggested by the ability of anti-schistosome anti-serum raised in rabbits to bind to host mouse serum molecules

Table 4 Host membrane and membrane-associated proteins obtained from proteomic analysis of isolated and enriched tegument surface membranes. Ref #1, Braschi *et al.*, 2006; Ref #2, Braschi and Wilson, 2006; Ref #3, Braschi and Wilson, 2006. Ref #3 yields a large number of protein hits: this work involved tegument differential extraction followed by fraction analysis by LC MS from a very large quantity of adult parasites. Accession numbers are from NCBI database

Accession number	Protein name	Ref #1	Ref #2	Ref #3
Host proteins				
gi 440121	IgG1 heavy chain		x	
gi 1304160	IgG3 heavy chain		x	
gi 70048	IgM heavy chain		x	
gi 28175786	Complement C3 fragment		x	
gi 41054731	Integrin alpha 2			x
gi 387438	C4 complement			x
gi 42543136	Crry complement regulatory protein Chain B			x
gi 229334	Fibrinopeptide B			x
gi 33636734	Chemokine (C-X-C motif) ligand 11			x
gi 11322386	Glycine receptor betaZ subunit			x
gi 191613	Acetylcholine receptor			x
gi 53674	CD44			x

(Damian, 1967). Immunodiffusion analysis suggested the existence of at least four antigens common to parasite and mouse (Damian, 1967).

The presence of host molecules on the parasite's surface was subsequently demonstrated by innovative worm transfer experiments. When adult worms were transferred from a mouse to a monkey that had been immunized against mouse red blood cells (an 'anti-mouse' monkey), most of the parasites did not survive the transfer. In contrast, worms transferred from mice to control, non-immunized monkeys (or to monkeys immunized against sheep red blood cells) survived well (Smithers *et al.*, 1969; Clegg *et al.*, 1970). These results were not always replicated when transfer experiments were carried out with other host species (Boyer and Ketchum, 1976) or different mouse strains (Boyer *et al.*, 1976). Nonetheless, the notion was established that host molecules could become attached to the parasite in the bloodstream and could (in this artificial circumstance) thereby provide a target for immune-mediated killing in the recipients immunized against donor host tissue, hence the term 'host antigen'.

Recovery of mouse worms from recipient monkeys at intervals after transfer revealed that by 25 hours the tegument was severely damaged with a vacuolated or destroyed syncytium (Smithers *et al.*, 1969), suggesting that most or all of the acquired host antigens were located at the tegumental surface. *In vitro*, anti-mouse antibodies plus complement damaged adult worms but did not kill them, while the addition of activated peritoneal cells resulted in worm death (Perez and Terry, 1973). The tegument was destroyed and the underlying muscle was exposed within two to four days. Ultrastructural analysis of worms under attack suggested that fragments of tegument membrane were being cast off and renewed from below. It appeared that "the schistosome is attempting to renew its surface membrane at a rapid rate to compensate for its destruction by antibody and complement" (Perez and Terry, 1973). Such worm transfer experiments also provided information about the time taken to shed acquired host antigens *in vivo*, indicative of the strength of their attachment to the parasite surface. All mouse worms transferred immediately to an 'anti-mouse' monkey were killed, while the bulk survived the procedure in a normal monkey. However, after residence in a normal monkey for three days, about half survived the transfer to an anti-mouse monkey and after 7 or 14 days, all survived. Although these data derive from small numbers of animals, they show that host antigen acquisition is not a permanent feature, with worms gradually losing their mouse antigens over a seven-day period, presumably exchanging them for monkey antigens (Smithers *et al.*, 1969).

When 15-day-old schistosomula were recovered from infected mice and transferred to monkeys immunized against mouse antigens (using either erythrocytes or spleen liver cells), all of the schistosomula are subsequently killed (Clegg *et al.*, 1971b). In contrast, some seven-day-old schistosomula (recovered from the lung tissue of infected mice) survived following transfer: no cercariae that undergo normal skin penetration are killed (Clegg *et al.*, 1971b). These data suggest that schistosomula take some time to acquire a sufficient density of host antigens (or must reach a particular developmental stage), to achieve protection against killing when transferred to an anti-mouse monkey (Clegg *et al.*, 1971b). However, schistosomula recovered from mouse lung tissue four days after infection, unlike

their three-hour counterparts from skin, bound anti-host erythrocyte antibodies to their surfaces (McLaren *et al.*, 1975). Moreover, by seven days, live *ex-vivo* lung schistosomula fluoresce strongly and evenly over the entire surface when reacted with antibodies to host erythrocyte ghosts (Riengrojpitak *et al.*, 1989). Freshly perfused adult worms have host molecules associated with their surfaces as determined using rabbit antisera to whole mouse extract in a mixed agglutinin test (Sell and Dean, 1972). In this assay, males were usually uniformly coated, whereas females were most strongly positive along approximately the anterior third of the body with the remainder being entirely negative, perhaps indicative of limited access by the females to host molecules while paired with males (Sell and Dean, 1972). Similarly, the surface of living adult worms stained 'strongly positive' when reacted with antisera to mouse erythrocytes, again indicating the presence of host antigens (Goldring *et al.*, 1977b).

5.2. Blood Group Antigens

The nature of the host molecules acquired by schistosomes in the bloodstream has been the subject of several indirect investigations that point to blood group antigens as the major fraction. Among these antigens, those of the ABO group have received most attention. Blood group O antigen terminates with the trisaccharide Gal(β ,1-4)[Fuc(α ,1-2)]GlcNAc; blood group A antigen links an α -GalNAc to C-3 of the Gal, whereas in the case of blood group B antigen a Gal is substituted for the GalNAc.

In worm transfer experiments, mouse erythrocytes or ghosts were found to provide greatest protection against mouse worms, but immunization with soluble proteins from mouse erythrocytes was also strongly protective (Clegg *et al.*, 1970). However, since immunization with mouse IgG provided substantially less protection against transferred worms, it was concluded that host antigen was not mouse IgG. Similarly, since immunization with red blood cells from one mouse strain provided cross protection against worms transferred from a different mouse strain, it was concluded that the protective antigens were not encoded by histocompatibility genes (Clegg *et al.*, 1970). It is

notable that MHC molecules and immunoglobulin were ruled out as potential major host antigens, yet there is evidence that precisely these molecules are present at the parasite surface (see Sections 5.5 and 5.8).

Schistosomula, incubated with soluble extracts of several different mouse tissues, subsequently stain with anti-whole-mouse antiserum suggesting that the parasites can acquire antigen from several sources (Dean and Sell, 1972), or that the host tissues contain erythrocytes that prime the immune system. Schistosomula, cultured with erythrocytes or erythrocyte extracts, can adsorb blood group A and B antigens onto their surfaces as determined by means of a mixed agglutination test (Dean, 1974). The antigens were detected on the parasites after 18–20 hour culture with intact erythrocytes, but after one hour exposure to alcohol extracts of erythrocyte membranes (Dean, 1974). It is telling that formalin-fixed, dead schistosomula can also acquire these antigens from red blood cells, showing that these substances are passively adsorbed. Since alcohol-soluble A and B antigens are known to be glycosphingolipids, it was suggested that a glycosphingolipid structure might be important in antigen binding to the parasite. No uptake of several other blood group antigens (H.M.N.P.C.D.E.P or Duffy) could be demonstrated (Dean, 1974). The fact that most of the other blood group antigens are proteins or glycoproteins suggests that protein transfer to the parasite surface is more difficult.

Further evidence that at least some host antigen epitopes are erythrocyte glycans was provided by culture of schistosomula for 15 days in the presence of erythrocytes of the AB Rh-blood group. Most parasites were destroyed (95–98%) when surgically transferred into the vasculature of Rhesus monkeys that had been immunized with the same erythrocytes in Freund's adjuvant, whereas controls were not, suggesting that the acquired host antigens were the targets of immune attack (Clegg *et al.*, 1971a). In later work, schistosomula were cultured for 15 days in a medium containing 50% blood from blood group A secretor, group B secretor or non-A secretor humans (Goldring *et al.*, 1976). The schistosomula were similarly transferred by surgery into the vascular system of monkeys immunized with blood type A glycoproteins in Freund's adjuvant. Those schistosomula that had been

cultured in type A blood were almost totally destroyed following transfer, while those cultured in B type blood survived well. Again, it was inferred that parasites cultured in type A blood had acquired A antigens on their surfaces which provided a focus for immune-mediated killing *in vivo* (Goldring *et al.*, 1976). Mixed agglutination and immunofluorescence analysis also provided evidence that not only A and B antigens, but also other glycan antigens such as H and Lewis^b + could be acquired by parasites during culture, whereas protein antigens such as Rhesus, M,N,S and Duffy antigen could not (Goldring *et al.*, 1976). Therefore, it was suggested that the host antigens were acquired in glycolipid form. Schistosomula cultured for three days in the presence of ³H-labeled human erythrocytes acquired small amounts of radioactively labeled low molecular weight material that was possibly lipid in nature (Goldring *et al.*, 1977a). Apparently no major glycoprotein components of the erythrocytes became associated with the schistosomula in these experiments (Goldring *et al.*, 1977a). In spite of this wealth of circumstantial evidence, the presence of specific host blood group molecules at the schistosome surface remains to be formally demonstrated by direct chemical analysis.

5.2.1. Forssman-Like Antigen

As noted earlier, schistosomula can be incubated with soluble extracts of several different mouse tissues and they subsequently stain with anti-whole-mouse antiserum (Dean and Sell, 1972). This has led to the conclusion that the parasites can acquire antigen from several sources. Absorbing the antisera with erythrocytes from species that possess the Forssman glycolipid antigen (but not from Forssman-negative species) eliminated the staining. The Forssman antigen is a ceramide pentasaccharide with the structure GalNAc(α ,1-3)GalNAc(β ,1-3)Gal(α ,1-4)Gal(β ,1-4)Glc ceramide. It was concluded that the parasites could adsorb from mice an antigen similar to but not identical to the Forssman antigen and that this antigen was also found on adult parasites (Dean and Sell, 1972). However, from worm transfer experiments, others have concluded that Forssman antigen is not among those acquired from the mouse host (Clegg *et al.*, 1970).

5.3. Skin Antigens

Patients suffering from the autoimmune disease *pemphigus vulgaris* develop antibodies to poorly defined 'intracellular substance' (ICS) of the epidermis. Using sera from such patients and immunofluorescence, schistosomula that penetrated mouse skin were shown to acquire ICS, while control schistosomula (mechanically transformed *in vitro*) did not (Smith and Kusel, 1979). Schistosomula recovered from the skin at later times following infection gained increasing quantities of the material. However, ICS could not be detected on six-day lung schistosomula, three-week parasites recovered from the liver or seven-week-old adult worms suggesting that any acquired ICS had been shed (Smith and Kusel, 1979).

5.4. Fibronectin

Anti-human fibronectin antibodies bound to the surface of adult parasites demonstrate a close association between fibronectin determinants and the outer *S. mansoni* membrane (Ouaissi *et al.*, 1984). Surface binding could be blocked by the presence of fibronectin. Additionally, this antiserum, in the presence of guinea pig serum (as a source of complement) was cytotoxic for lung schistosomula and adult worms. Treated parasites had a disrupted outer membrane and a degenerate, vacuolated underlying cytoplasm (Ouaissi *et al.*, 1984). Fibronectin determinants were not revealed on the surface of skin schistosomula, nor were these parasites damaged in the cytotoxic assay just described (Ouaissi *et al.*, 1984). An involvement of surface fibronectin in parasite adhesion to the vascular endothelium or in the binding of complement components has been proposed (Ouaissi *et al.*, 1984). Sequences encoding proteins with fibronectin domains are present in the *S. mansoni* transcriptome and genome (Sm27904); a peptide hit to a protein with fibronectin domains (snap 20471) was also obtained by LC MS in a Tris extract of a highly enriched tegument membrane preparation (S. Braschi, personal communication) but *bone fide* host fibronectin was not detected.

5.5. MHC Molecules

S. mansoni schistosomula, recovered from the lungs of inbred mice, were shown to possess serologically detectable alloantigens on their tegumental surfaces (Sher *et al.*, 1978). Alloantigen sera were prepared by cross-immunizing C3H/HeJ and C57BL 6J mice using pooled spleen and lymph node cells in Freund's adjuvant as the immunogen. All lung schistosomula (10/10) recovered from C3H mice stained positively with the C57 anti-C3H antiserum; none stained with the C3H anti-C57 antiserum. Likewise, all parasites (10/10) recovered from C57 mice stained positively with the C3H anti-C57 antiserum and not with the C57 anti-C3H antiserum (Sher *et al.*, 1978). Using congenic mice and appropriate antisera, gene products of the K and I sub regions of the major histocompatibility complex were demonstrated among those alloantigens acquired by the parasite. The K and I regions encode MHC class I and MHC class II glycoproteins. These data extended the list of host molecules associated with the schistosome surface from glycolipids to include glycoprotein. The acquisition of these molecules is selective since parasites were found not to stain for other known cell surface antigens including Thy 1, Ly 1 and H-Y (Sher *et al.*, 1978). Likewise, mouse albumin, immunoglobulin and complement component 3 could not be detected on the surface of lung-stage schistosomula by immunofluorescence (Sher *et al.*, 1978).

Schistosomula recovered from the lungs of mice and reinjected into allogeneic recipients were shown to exchange their alloantigens. This exchange process had begun to occur by 15 hours after reinjection, and at the termination of the experiment (87 hours after reinjection) all of the parasites expressed surface alloantigens of their new hosts. However, worms staining positively for alloantigens of their original hosts could also be demonstrated at the 87-hour time point (Sher *et al.*, 1978). Schistosomula also acquired alloantigens when cultured with lymphoid cells *in vitro*, whereas no uptake was evident when allogeneic serum was used as a source of host antigen (Sher *et al.*, 1978).

Monoclonal antibodies recognizing H-2K^b bind to the surface of lung-stage schistosomes (Gitter and Damian, 1982; Simpson *et al.*, 1983b). Furthermore, these antibodies precipitate a ~45-kDa molecule

from surface-labeled lung-stage parasites and a molecule of a similar size from labeled spleen cells (Simpson *et al.*, 1983b). Using a variety of anti-alloantigen antisera, gene products from other regions of the MHC region have been demonstrated on the surface of lung-stage parasites (Gitter and Damian, 1982). These include products from the H-2D^k, I-E^k, H-2K^b, H-2D^b and I-A^b regions (Gitter and Damian, 1982).

The presence of MHC molecules on the parasites does not induce allogeneic spleen and lymph node cells to respond and proliferate in a reaction analogous to a primary mixed lymphocyte reaction (MLR) (Gitter *et al.*, 1982). In an MLR, donor T cells with specificity directed against alloantigens on recipient cells are triggered to proliferate in the presence of these antigens. Alloreactive cytotoxic T lymphocytes can adhere to alloantigen-bearing lung-stage parasites (Butterworth *et al.*, 1979). Nevertheless, these parasites remain fully viable, even after prolonged incubation (Butterworth *et al.*, 1979).

Live adult parasites were treated with alloantiserum, specific for an H-2K^k gene product. Since FITC-labeled *Staphylococcus aureus* bound to the tegumental surface of these parasites, and not controls, this suggested that the adult parasites retained the ability to acquire host MHC determinants (Gitter *et al.*, 1982).

In order to determine whether the MHC molecules are acquired by the parasite from the host or are synthesized by the parasite, cloned HLA cDNA was radiolabeled and hybridized to schistosome (cercarial and adult) DNA. Since no hybridization was detected between the two, this indicates that there are no DNA sequences homologous to class I MHC antigens in the parasite genome (Simpson *et al.*, 1983b). This is consistent with the notion that the parasite has acquired the host MHC proteins. More recently, a set of short 121 bp DNA fragments, with 89–100% sequence identity to a region of the mouse Class I MHC sequence (H2-Q1), were identified by PCR in the *S. mansoni* genome. These were suggested to have been transmitted from the host or to have evolved to mimic host MHC sequence, although there is as yet no evidence that protein is derived from this sequence (Imase *et al.*, 2001), and no MHC proteins have been detected by mass spectrometric analysis of schistosome tegumental membranes (Braschi and Wilson, 2006; Braschi *et al.*, 2006).

5.6. α 2-Macroglobulin

α 2-Macroglobulin is a potent proteinase inhibitor found in the blood-stream. Three of seven rabbits immunized with parasite homogenates in Freund's adjuvant generated antibodies that reacted with mouse α 2-macroglobulin (Damian *et al.*, 1973). Mixed agglutination reactions suggest that the antisera bound to determinants with an uneven distribution on the surface of male parasites; females were rarely agglutinated (Damian *et al.*, 1973). This result was confirmed using immuno-electron microscopy; anti-mouse α 2-macroglobulin bound with a patchy distribution to surface membranes and tegumental infoldings of live or fixed adult parasites using this technique (Kemp *et al.*, 1976b). The antisera reacted with parasites obtained from Rhesus monkeys as well as those of murine origin. Whether parasites synthesize α 2-macroglobulin-like surface molecules or acquire the hosts α 2-macroglobulin has not been determined.

In order to ascertain whether α 2-macroglobulin was the target of protective immunity in the worm transfer experiments described earlier, Rhesus monkeys were immunized with purified mouse α 2-macroglobulin in Freund's adjuvant, and subsequently challenged with mouse worms surgically implanted into their hepatic portal vasculature. These monkeys did not show an increased ability to destroy the mouse worms and the worms themselves were unaffected by the presence of fairly high levels of circulating anti-mouse α 2-macroglobulin antibodies (Damian *et al.*, 1973). Furthermore, no host α 2-macroglobulin has been detected in the tegumental proteome by MS (Braschi and Wilson, 2006; Braschi *et al.*, 2006).

5.7. Complement Factors

5.7.1. Delay Accelerating Factor

Adult worms and schistosomula are refractory to the damaging effects of complement. Trypsin treatment of these parasites converts them to complement sensitivity (Marikovsky *et al.*, 1990). This suggests that trypsin cleaves complement regulatory protein(s) from the

parasite surface. Antibodies raised against the trypsin-released material bind to the parasites and induce their killing via complement (Marikovsky *et al.*, 1990). The ability of worms to evade activation of the alternative complement pathway has been linked to the presence of DAF or a related molecule, on the surface membranes (Pearce *et al.*, 1990). Such a molecule could inhibit the activity of complement components, C3 and C5 convertase, and ultimately prevent the buildup of complement membrane attack complexes. Antibodies against human or guinea pig DAF bind to the surface of worms recovered from Rhesus monkeys and immunoprecipitate a 70-kDa surface molecule from surface-radiolabeled schistosomes. PIPLC-treated schistosomula bind anti-DAF antibody significantly less well than non-treated controls (Pearce *et al.*, 1990). The host DAF molecule was suggested to have been incorporated by its hydrophobic diacylglycerol moiety into the outer lipid bilayer of the parasite (Pearce *et al.*, 1990).

Schistosomula incubated with normal human erythrocytes (but not DAF-deficient erythrocytes) become resistant to complement-induced damage *in vitro* (Horta *et al.*, 1991). Additionally, these parasites acquire a 70-kDa surface protein that can be immunoprecipitated with anti-DAF antibodies (Horta *et al.*, 1991). It is possible that transfer of DAF from the host to the parasites helps prevent complement-mediated damage. No DAF molecules have been detected in the tegumental proteome by MS (Braschi and Wilson, 2006; Braschi *et al.*, 2006).

5.7.2. C3c/C3dg

The alpha chain C3c/C3dg fragment of complement factor C3 can be biotinylated and detected by proteomics at the tegument surface of live worms (Braschi and Wilson, 2006). This implies that C3 can be activated by C3 convertase, covalently linked to the surface, and subsequently inactivated by the complement regulatory protein (CRP) Factor I in combination with membrane cofactor protein (MCP) or Factor H. DAF, mentioned above and identified at the schistosome surface by immunocytochemistry (Pearce *et al.*, 1990),

acts too early by accelerating the destruction of C3 convertase, to explain the presence of the C3c/C3dg fragment. However, murine Complement receptor-related protein y (Crpy), which possesses both DAF and MCP activity, was detected in an LC-based tandem MS analysis of purified tegument membranes (S. Braschi, personal communication). The detection of an inactive form of C3 at the parasite surface argues that schistosomes may inhibit the complement pathway by the recruitment of CRPs (like Crpy) from host plasma.

5.8. Immunoglobulin

The ability of adult parasites to adsorb heterospecific antibody onto their tegumental surfaces was shown by several groups, though occasionally with conflicting results. It has been suggested that host IgG, bound to the parasite surface via their Fc regions, might sterically hinder parasite-specific antibody from binding to appropriate antigens (Tarleton and Kemp, 1981). Further, antibody bound in this way would be unable to fix complement.

In the first report of this nature, adult worms recovered from baboons were washed and incubated in rabbit anti-baboon IgG antisera. This antibody was detected over all parasite surfaces immunohistochemically, thus demonstrating the presence of IgG on the adult tegumental surface (Kemp *et al.*, 1976a). In another experiment, infected mice were first immunized against red blood cells or bovine serum albumen or horseradish peroxidase. Next, worms recovered from those animals and incubated in their respective antigens selectively bound to their tegumental surfaces only those antigens to which their murine host had been immunized (Kemp *et al.*, 1977). Antigen binding was not uniform and accounted for a very small proportion of the parasites total surface, but was heaviest in the extreme dorsal anterior and posterior of the male worm (Kemp *et al.*, 1977). In other work, schistosomula were incubated with mouse anti-sheep red blood cell antibody and then with sheep red blood cells. In a process called rosetting, the erythrocytes coat the parasites. The conclusion was that antibody bound to an Fc receptor at the parasite surface, leaving antigen-binding sites available to adhere

to the red blood cells (Torpier *et al.*, 1979a). Five-day-old schistosomula and adult worms no longer expressed the ability to form rosettes, suggesting (in contrast to the work presented above (Kemp *et al.*, 1977)) the absence of Fc receptors in these more mature parasites.

The specific antibody isotypes involved in binding to the parasite surface was investigated. Fluoresceine-labeled anti-IgG2b antibody stained the surface and tegument of adult parasite sections 'apple green' (Sogandares-Bernal, 1976), suggesting the presence of this antibody isotype at the surface. However, IgA, IgM, IgG1, IgG2a and IgG3 were not similarly detected although why these would not bind through their Fc domains is unclear (Sogandares-Bernal, 1976). In contrast, IgG1, IgG2a and IgG2b were all detected by other workers, with a general, homogeneous distribution on the outer tegumental membrane of adult parasites, using immunocytochemistry (Kemp *et al.*, 1978). The distribution of IgG3 at the surface is described as being 'in quantity but spotty' (Kemp *et al.*, 1978). IgA and IgM were also detected but in distinct and separate aggregates. The presence of each of these murine immunoglobulin isotypes at the surface of worms recovered from mice was confirmed using anti-isotype specific antisera that were detected by their association with fluoresceinated *S. aureus* (Kemp *et al.*, 1980). The *S. aureus* surface protein, protein A, binds to the Fc region of immunoglobulin. Antibodies were also detected in parasite eluates by immunodiffusion (Kemp *et al.*, 1978).

Further evidence for the presence of IgG Fc receptor on the adults was later presented using an indirect, amplification assay (Tarleton and Kemp, 1981). Attempts to visualize Fc receptors directly, using labeled immune complexes were not successful, suggesting a paucity of such receptors or that the receptors on recovered parasites already contain bound immunoglobulin (Tarleton and Kemp, 1981). The inability of an Fc-binding reagent (fluoresceinated *S. aureus*) alone to bind to the parasites in this study suggests that no anti-parasite-antigen antibody (with its exposed Fc domain) is bound to the recovered parasites (Tarleton and Kemp, 1981). This suggests that *in vivo* the tegument is not coated with anti-schistosome immunoglobulin and therefore might not be a focus of active humoral immunity.

In later attempts to identify host molecules associated with the adult parasites, worms were first recovered from infected mice and cultured *in vitro*. Material released by these adult parasites was concentrated and resolved by SDS-PAGE. Several antibody classes (IgG, IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) were detected in the concentrate by Western analysis, using specific antisera (Gearner and Kemp, 1994). Mouse albumin and α 2-macroglobulin were similarly detected. In contrast, specific antisera against mouse erythrocytes, hemoglobin, C3, transferrin, fibronectin, fibrinogen and MHC protein H-2Db heavy chain did not react with any component (Gearner and Kemp, 1994).

Note that the reported level of detection of bound immunoglobulin varies from 'general and homogeneous' (Kemp *et al.*, 1978) to non-existent (Sher *et al.*, 1978; Torpier *et al.*, 1979a). This is in keeping with the ability of some to detect Fc receptor (paramyosin) at the surface (Loukas *et al.*, 2001) and the inability of others to do so (Davies and Pearce, 1995; Skelly and Shoemaker, 1996; Braschi and Wilson, 2006) and suggests, as noted earlier, that Fc receptor accessibility may vary depending on parasite strain, parasite age or culture and experimental conditions.

In tegumental proteome studies, immunoglobulin isotypes IgM, IgG1 and IgG3 can be biotinylated on the surface of freshly perfused adult parasites (Table 4) (Braschi and Wilson, 2006) and identified by tandem MS. Whether these antibodies are bound to the surface via their Fab or Fc domains remains unresolved.

5.9. Possible Mechanisms of Host Molecule Acquisition

A number of mechanisms have been proposed to explain how the parasites acquire host molecules onto their surfaces. One such mechanism is through the physical fusion of the parasite surface membrane with the plasma membrane of host cells. Evidence for the possibility of such membrane fusion was obtained following the incubation of schistosomula with neutrophils in the presence of serum or complement (Caulfield *et al.*, 1980a, b). It is postulated that IgG and C3, bound to the surface of the parasite, interact with receptors on the

host cell membranes to bring parasite and host very close together (Caulfield *et al.*, 1980a). By freeze fracture techniques, points of contact were observed to consist of fusions of the outer leaflets of the neutrophil with the tegumental membranes. Such fusion of host and parasite may preempt exocytosis of the neutrophil granule contents by preventing fusion of the perigranular membrane with the plasma membrane (Caulfield *et al.*, 1980a). However, the phenomenon does not appear very common in this system; schistosomula are incubated with neutrophils at a ratio of 1:2000, yet only 50% of the parasites have 20 or more neutrophils adherent at the end of the incubation period (Caulfield *et al.*, 1980a). It has been pointed out that the morphology of fusion may represent an artifact of tissue preparation (Caulfield *et al.*, 1980a). No fusion of eosinophils to the parasites was observed (Caulfield *et al.*, 1980b). Furthermore, this notion of cell fusion with the surface as a mechanism of host molecule acquisition does not explain the selectivity of molecules that are acquired; as mentioned earlier, only certain blood group antigens are detected, for instance, and not others. Instead of whole cell fusion, it has been suggested that perhaps membrane vesicles bleb from the surface of mammalian cells and fuse with the parasite surface, or host molecules are acquired that have been shed as a result of normal cellular turnover (Pearce *et al.*, 1990).

A second potential mechanism of host molecule acquisition is through the enzymatic cleavage of the exposed hydrophilic portion of a host molecule and its transfer to the parasite surface (Simpson *et al.*, 1983b). However, the analysis of parasite-acquired MHC molecules suggested that more than just the hydrophilic region was transferred (Simpson *et al.*, 1983b). The fact that blood group antigens can be acquired by both living and formalin-fixed schistosomula provides the strongest argument that some at least of the host antigens are passively adsorbed (Dean and Sell, 1972). Consequently we believe that the acquisition of host molecules is simply due to the hydrophobicity of the parasite's membranocalyx. Parasites may acquire compatible molecules that they are in direct physical contact with in a given location. Thus, migrating schistosomula acquire skin antigens in the skin, MHC molecules when squeezing through the lung microvasculature, and erythrocyte glycolipids throughout the blood stream.

5.10. Functional Significance of Host Molecule Acquisition

The most attractive hypothesis to explain parasite acquisition of host molecules is that they provide a disguise for the pathogen. This might either prevent recognition of the parasite by immunological surveillance and thereby the induction of anti-parasite immunity, or mask its surface proteins from attack by immune effector mechanisms. It has proved difficult to design an *in vivo* experiment that would test the absolute requirement for host antigens. Indeed, it may be that their acquisition has no functional significance for the parasite but is merely a consequence of the chemical and biophysical nature of its outer surface.

There is ample evidence that after transformation from cercariae, schistosomula become progressively more resistant to immunological effectors, and older schistosomes express little exposed parasite antigen (McLaren *et al.*, 1975; Goldring *et al.*, 1977b; Bickle and Ford, 1982; Pearce *et al.*, 1986a). This resistance to immune killing *in vitro* is accompanied by an increase in the ability of antisera to host erythrocytes to bind to the parasites (i.e. to detect acquired host molecules) and a decrease in the ability of serum from chronically infected mice (presumably detecting parasite antigens) to similarly bind (Goldring *et al.*, 1977b; Bickle and Ford, 1982; Harnett *et al.*, 1985; Harnett and Kusel, 1986). Such data could be interpreted as evidence that the host molecules are masking parasite antigen, hindering anti-parasite immunity.

However, unlike fresh three-hour schistosomula, those cultured for 24–48 hours in medium lacking serum and macromolecules are not susceptible to antibody, complement or eosinophil-mediated killing (Dessein *et al.*, 1981). This suggests that the acquisition of host antigens is *not* required for the resistance of these parasites to immune-mediated damage. This resistance is related to the simultaneous reduction in the ability of human anti-schistosomula antibodies and human complement component C3 to bind to the surface of the cultured larvae (Dessein *et al.*, 1981). Others have likewise shown that schistosomula cultured in the absence of macromolecules are resistant to cytotoxic killing by anti-schistosomula antibodies plus complement

(Dean, 1977). Yet other workers report that the *presence* of host serum significantly enhances resistance to antibody or cell-mediated parasite killing (Tavares *et al.*, 1978, 1980; McLaren and Incani, 1982). Nonetheless these authors also suggest that resistance involves modulation of the parasite surface rather than masking of parasite antigens by acquired host antigens (Tavares *et al.*, 1978; McLaren and Incani, 1982). The added serum is suggested to promote surface modulation (Tavares *et al.*, 1978). Indeed, the ability to detect parasite antigen is lost before appreciable amounts of host molecules are acquired. This suggests that the diminishing exposure of parasite antigens that accompanies larval development is not due to their masking by host molecules (Pearce *et al.*, 1986a).

6. CHANGES AT THE SURFACE DURING PARASITE MATURATION

We noted earlier the distinct differences in tegument composition between the newly transformed schistosomulum and the adult worm. Some researchers have attempted to characterize the molecular modifications occurring at the surface of the schistosome tegument during development from cercaria to adult. Parasite surfaces were iodinated and labeled antigens were immunoprecipitated with a range of antisera (Payares *et al.*, 1985c). A set of 32–38 kDa glycoproteins was identified at the cercaria and schistosomula surfaces. Low molecular weight antigens (15, 17 and 20 kDa) were additionally identified in schistosomula. With maturation, the 32–38 kDa set was replaced by a single dominant 32-kDa glycoprotein. The 15 and 20-kDa antigens were also detected through to the adult stage and new antigens were also detected (25 and 97 kDa, the latter possibly being paramyosin) (Payares *et al.*, 1985c). Other researchers have immunoprecipitated surface-iodinated proteins of similar molecular weights (40, 37 and 32 kDa) from schistosomula using serum from infected rats or humans (Dissous and Capron, 1981). Additionally, three similar proteins (38, 32 and 18 kDa) were shown to be GPI anchored at the surface of cercariae and schistosomula (Pearce and Sher, 1989). In an earlier study, dominant, adult parasite, surface-labeled proteins of

different Mr were immunoprecipitated (at > 150, 78, 45 and 22 kDa), using serum from a rabbit immunized with an adult worm freeze thaw fraction (Shah and Ramasamy, 1982). The identity or the relatedness of these labeled surface molecules is largely unknown, although tryptic and chymotryptic digests of the 20, 32 and 38 kDa surface-labeled proteins suggests that all were related (Payares *et al.*, 1985a). One 65-kDa band, seen at the surface of lung worms and later developmental stages, was shown to be alkaline phosphatase (Payares *et al.*, 1985c).

While immature parasites (either three-hour cultured schistosomula or five-day lung worms or three-week-old worms) were readily radio-labeled, iodination of surface proteins on intact, six-week-old adults was accomplished only with difficulty (Snary *et al.*, 1980; Payares *et al.*, 1985c). A tegument-enriched fraction was recovered, radioiodinated and then immunoprecipitated. The identification of common antigens in these labeled purified membranes from adult worms demonstrated that at least three molecules (15, 20 and 32 kDa), exposed on the surfaces of the younger stages, were present but not exposed at the surface of intact adult worms. It was concluded that these antigens had not disappeared or been shed from the adult tegument but were sequestered deeper into the surface membrane complex, perhaps aiding the parasite's ability to evade immune damage (Payares *et al.*, 1985c). Similarly, whereas GPI-anchored proteins could be enzymatically released from the surface of three-hour cultured schistosomula, none was detectable after treatment of seven-day-old lung-stage parasites (Pearce and Sher, 1989). However, Sm200 is recoverable from PIPLC-treated adult worms (Sauma *et al.*, 1991).

These data suggest differences in tegumental membrane structure and composition in intra-mammalian parasites of different ages, which in turn implies changes in the composition of tegumental secretory inclusions. Thus, one explanation for the apparent masking of parasite antigens is that new multilaminar bodies, containing lower densities of protein, fuse at the surface of the growing parasites, a process that could also alter the composition of the plasma membrane (Payares *et al.*, 1985c; Pearce *et al.*, 1986a). Likewise, the inability to label the surface of older parasites or to detect parasite antigen at the surface, noted above, may be due to the biogenesis of modified tegumental membranes (perhaps including a more

label-impermeable membranocalyx) (Snary *et al.*, 1980; Payares *et al.*, 1985c). Certainly, differences in tegumental lipid composition have been documented as the parasites mature (see Section 3). It is noteworthy that the tegument cytoplasm of lung schistosomula contains a unique secretory inclusion, the homogeneous body (McLaren *et al.*, 1978; Crabtree and Wilson, 1986), of unknown function, although a role for its contents in lubricating the passage of schistosomula along capillaries was suggested (Crabtree and Wilson, 1986).

We favor the notion that the membranocalyx may trap host macromolecules as the parasites migrate. Invading parasites acquire blood group or tissue antigens. We hypothesize that the membranocalyx (with its acquired host moieties) yields a boundary layer that minimizes the approach of both host antibodies and cells. Similar to a bacterial glycocalyx, the membranocalyx of schistosomes may afford protection from the immune response by a variety of mechanisms, including decreased cellular adhesion and protection from the effects of complement. These changes in antigen accessibility, combined with other active evasion strategies, may likewise lower the immunological accessibility of the external surface of developing schistosomes *in vivo*.

7. TEGUMENTAL MEMBRANE DYNAMICS

The model adopted in this review is of a tegument outer surface bounded by a plasma membrane overlain by a secreted membranocalyx. This architecture is maintained by the generation of multilaminate vesicles in the tegumental cell bodies and their ultimate fusion with the surface to contribute simultaneously both plasma membrane and membranocalyx. Since the surface area of the lamellate contents of a vesicle is about twice that of its bounding membrane, it implies that the membranocalyx turns over that much quicker. As in other secretory systems, it is implicit in the model that the membranocalyx would be shed into the bloodstream, while the plasma membrane would be internalized back to the Golgi apparatus or endosomes. The key to immune evasion by schistosomes lies in an understanding, not just of the composition but also the dynamics of this complex surface. Nonetheless, after more than three decades of investigation we still

have a very imperfect grasp of tegument surface dynamics, not least because schistosomes present a difficult experimental system. One issue is how best to relate experimental results obtained using parasites outside of their hosts with conditions *in vivo*. Morphological changes in the tegument (and elsewhere) are sometimes observed following adult schistosome culture in a variety of media, as documented using both SEM and TEM (Wilson and Barnes, 1974a; Rumjanek and McLaren, 1981; Carlisle *et al.*, 1983). This highlights the difficulty of interpreting experiments that involve any assessment of tegumental turnover or function *in vitro*. The robustness of the tegument *in vivo* contrasts with its extreme fragility *in vitro*. The recovery of adult worms and their *in vitro* handling all too readily leads to surface damage and the exposure/unmasking of subsurface proteins, making the task of understanding function and dynamics all the more difficult.

7.1. Biogenesis

Attempts have been made to assess the dynamics of biosynthesis and export of tegument constituents using radiolabeled precursors (amino acids, monosaccharides and fatty acids). It should be noted that in these studies, the incorporation of label into parasite fractions was determined, rather than authenticated constituents of the surface membrane complex. With our current knowledge of surface composition, these experiments would repay repeating. A second caveat for the protein and oligosaccharide labeling is the necessity of performing the labeling pulse in medium lacking supplements (e.g. in Hank's balanced salt solution) to ensure a high specific activity of incorporation, with a potential reduction in worm viability as a consequence.

Adult parasites cultured *in vitro* for 24 hours have been proposed to release particulate membrane antigens, since the medium contains membranous material, as determined from examination by electron microscopy (Kusel *et al.*, 1975). Whether the released material represents true surface turnover or is a byproduct of culture conditions is

not clear. Surface membrane extracts prepared from adult worms labeled first with ^{14}C leucine and then with ^3H leucine were resolved by SDS-PAGE and the ^{14}C : ^3H ratio determined for the various bands (Kusel and Mackenzie, 1975). Since the ratio remained virtually unchanged for the majority of bands, it was concluded that most of proteins at the surface turned over at about the same rate (Kusel and Mackenzie, 1975).

A second study used autoradiography to track the incorporation of 30-minute pulses of ^3H leucine into proteins and ^3H glucosamine into oligosaccharides, and the subsequent translocation of labeled macromolecules during chase incubation (Wilson and Barnes, 1979). It revealed that the major synthetic tissues of the adult male worm were the tegument cell bodies and gut epithelium. At the end of the 30-minute pulse, the bulk of labeled protein was in the tegument cell bodies, but some had already passed to the tegument syncytium. The concentration of labeled protein reached a maximum in the tegument after 1.5-hours chase incubation, and by three hours half had been lost either through secretion into the culture medium or turnover in the tegumental syncytium. The rates for oligosaccharide appeared to be of the same order as for proteins, suggesting the existence of a rapid secretory process in the tegument.

The turnover of adult tegumental phospholipids was examined by tracking the incorporation of the labeled palmitic acid into cultured parasites. Relative to the phospholipid of the internal tissues, tegumental phospholipids appeared metabolically very active, since label was first incorporated and subsequently lost more rapidly (within 20 hours) (Brouwers *et al.*, 1999). No labeled membrane phospholipid material was recovered from the medium, suggesting that membrane was not being rapidly shed. Instead, the rapid loss of label was due to the hydrolysis of labeled acyl chains. Deacylation/reacylation reactions were confined to the phosphatidyl choline fraction suggesting that the turnover rate of this phospholipid is higher than that of the phosphatidyl ethanolamine fraction (Brouwers *et al.*, 1999). Such rapid turnover has been proposed to expose adherent host cells to lysophosphatidylcholine and free fatty acids and possibly destabilize those (Brouwers *et al.*, 1999).

7.2. Tegument Membrane Fluidity

A number of studies have attempted to characterize the fluidity of the tegument surface membranes as a means to understand surface dynamics. Where labels have been attached externally, it is reasonable to assume that they are associated with the membranocalyx. Where lipophilic probes are involved, it is not clear whether they insert into the membranocalyx, the plasma membrane or both. The rapid lateral diffusion of lectin-coated or antibody-coated tegumental membrane, indicating its highly fluid nature, has already been noted (Torpier *et al.*, 1979b; Torpier and Capron, 1980). In contrast to these results, other workers have labeled adults with fluorescinated lectin probes (Con A, WGA) or Dil-C₁₈-(3) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate to label lipids), and applied a pulse of laser light to bleach the fluorescence at one area (Johnson *et al.*, 1982). The 'recovery' of label into the area was then measured as an indicator of fluidity. In this system, recoveries were 'remarkably incomplete' with the lipid probe being effectively immobile, suggestive of some degree of membrane rigidity (Johnson *et al.*, 1982). However, certain regions of the parasite (e.g. behind the oral sucker) are reported to give fast and almost complete recovery, suggesting that the properties of the parasite surface may vary with anatomical location (Johnson *et al.*, 1982). Incomplete recovery of a second fluorescent lipid analog, C₁₈F1 (5-(octadecanoyl)-amino-fluoresceine), further suggests that the adult surface membrane is differentiated into domains of different lipid composition or structure (Foley *et al.*, 1986). Regional differences in surface 'adhesiveness' have also been reported (Podesta *et al.*, 1987).

Two lipid compounds, BODIPY FL ceramide (*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosine and PKH2 pass rapidly through the outer surface of adult male worms *in vitro* and enter structures in or below the membranes (Redman and Kusel, 1996). This suggests the existence of membrane regions that permit such non-polar lipids to pass through. Two other fluorescent lipid compounds -5-(*N*-octadecanoyl)aminofluoresceine (AF18) and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl) sphingosylphosphocholine (BODIPY FL sphingomyelin) instead insert into the surface membranes of the adults. The diffusion rate

of these two lipids in the surface differ substantially (Redman and Kusel, 1996). The data suggest that lipid uptake is selective and, once within the bilayer, lipids may be sequestered into specialized micro-domains with different properties. Different membrane domains may have specialized functions such as the sequestration of selected proteins (perhaps GPI-anchored proteins, for instance) or to promote nutrient uptake or signaling.

Adult tegument fluidity is altered by incubating the parasites with serotonin, whose receptors are coupled to adenylate cyclase (Sands and Kusel, 1992). The fluidity of the fluorescent lipid membrane probe 5AF (5-*N*'-octadecanoyl aminofluorescein) is decreased following serotonin treatment but not so the fluorescent phospholipid NBD-PC (*N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-phosphatidyl choline) (Sands and Kusel, 1992). This suggests that the two probes have partitioned into two separate membrane domains that are controlled differently and further emphasizes the fact that the surface membrane is composed of micro-domains of heterogeneous composition that differ in their biochemical and physical properties.

A comparison of the mobility of several lipid analogs in parasite versus host cell membranes reveals that the rate of diffusion is an order of magnitude lower than the values typically obtained for host cell membranes (Foley *et al.*, 1986). Thus, the parasite presents a markedly different membrane surface to the host in terms of the diffusion characteristics of its molecules. In addition, the diffusion rate for immature forms is about 10-fold less than that of the adults (Foley *et al.*, 1986). One potential immunological consequence of this may arise if surface epitopes are separated in relatively immobile membrane domains. The inability of such epitopes to diffuse may limit the capacity of divalent antibody to bind to them (Kusel and Gordon, 1989). This raises the question as to how the proteins in the membranocalyx might be immobilized by the tegumental cytoskeleton given the existence of the intervening plasma membrane.

In the context of membrane domains, the presence of caveolae-like structures in the adult schistosome surface membrane has been described (Racoosin *et al.*, 1999). Caveolae are specialized invaginations of the plasma membrane enriched in certain lipids, cholesterol and protein (Anderson, 1998). An NP-40 detergent-insoluble fraction of

a schistosome surface membrane-enriched preparation was obtained. The fraction contained several GPI-linked proteins including alkaline phosphatase, Sm200 and Sm23. In addition, a schistosome protein, recognized by anti-human caveolin antibody, was detected in the detergent-insoluble fraction. Using electron microscopy, caveolae-like structures were observed in the surface membrane preparations (Racoosin *et al.*, 1999). Antibodies against the fraction immunolabeled tubercles on the dorsal tegument of adult worms, but this could be indicative of tegument surface abrasion. Anti-alkaline phosphatase antisera similarly labeled tubercles. By analogy with the role of caveolae in mammalian cells, the caveolae-like structures described in the schistosome surface membrane may serve as foci for signaling or metabolic uptake molecules (Racoosin *et al.*, 1999). However, caveolin itself has not been detected in either the *S. mansoni* transcriptome or genome (Verjovski-Almeida *et al.*, 2003).

7.3. Membranocalyx and Plasma Membrane Turnover

When the double bilayer structure of the tegument surface was first established, it appeared to provide a mechanism of immune evasion whereby any host antibody or complement factor binding to the surface could be shed by rapid turnover of the membranocalyx (Wilson and Barnes, 1977; Zhou and Podesta, 1989; Sauma *et al.*, 1991; Abath and Werkhauser, 1996). An alternative hypothesis would be that the parasite rapidly endocytosed bound immunological effector molecules but no convincing evidence for endocytosis at the schistosome tegument surface has been presented (Redman *et al.*, 1997). For example, endocytosis of labeled reagents bound to, or associated with, the surface (for instance, labeled LDL) has not been observed (Bennett and Caulfield, 1991). Indeed, the presence of the membranocalyx makes impossible extrinsic labeling of the plasma membranes with probes conventionally used to demonstrate endocytosis.

The first attempt to measure membranocalyx turnover involved the attachment of cationized ferritin non-covalently to the negatively charged surface in a 30-minute pulse incubation. The rate of loss of the label was then followed by chase incubation in medium, using

electron microscopy (Wilson and Barnes, 1977). By four hours most of the label had been lost, indicating a half-life of two to three hours. It was also apparent from the electron micrographs that sheets of membranocalyx with attached ferritin moved over surface, up the sides of spines, and were shed. In subsequent experiments, similar rates of turnover were obtained when adult parasites were surface labeled with ^{125}I and the rate of loss of label was monitored in the presence or absence of cationized ferritin. A half-life ($t_{1/2}$) for the surface label of 3.5 hours was noted in the presence of cationized ferritin, and a $t_{1/2}$ of six hours in the absence of ferritin (Roberts *et al.*, 1983). However, it should be noted that the bulk of label was attached to lipids rather than proteins.

The suggestion of rapid turnover is also seen when lectin (ConA or WGA) binds to living adult parasites. Intramembrane particles (IMPs, assumed to be integral membrane proteins and/or membrane-associated complexes) aggregate rapidly in the outer tegumental membrane, as observed by freeze fracture analysis (Torpier and Capron, 1980). Within five minutes of incubation with lectin, the migration of these particles to the tegumental spines is detected as a 'capping' of the ligand IMP complex. Such rapid lateral diffusion indicates a high fluidity of the membranocalyx (Torpier and Capron, 1980). Antibody binding to the surface of adult worms results in a similar membrane reorganization to that described above for lectin binding. Incubation of parasites in immune sera (containing antibodies against host molecules) results in a clustering of IMPs on the external membrane, as determined by freeze fracture analysis (Torpier *et al.*, 1979b). As early as 30 minutes following incubation, IMP clusters accumulate in the membranes of the surface tegumental spines and suggest a rapid release of immune complexes from the surface (cf. Wilson and Barnes, 1977; Torpier *et al.*, 1979b).

In a similar manner, others report that the binding of immune complexes to cultured parasites (to demonstrate the presence of Fc receptors and C3 receptors) was only possible in the presence of metabolic inhibitors when the incubations are carried out at 37 C, presumably to prevent bound immune complexes (and or their receptors) from being rapidly shed from the parasite surfaces (Tarleton and Kemp, 1981). Reports suggest that bound immunoglobulin can

be shed from cultured parasites; IgG has been detected in biotinylated surface extracts of fresh parasites but in a much lower level in extracts of parasites cultured for 24 hours, suggesting the immunoglobulin has been shed over this time period during *in vitro* culture (Loukas *et al.*, 2001). When bound by a ligand such as homologous antigen or anti-immunoglobulin, host immunoglobulin is reported to be shed rapidly --within 20 minutes at 37 C (Kemp *et al.*, 1980). When fresh, living parasites are recovered from mice and incubated with anti-mouse isotype-specific antibody (anti-IgG1, for instance) for 20 minutes at 37 C, the IgG1 can no longer be detected and is presumed to have been shed. However IgG2a, 2b, 3, A and M remain clearly detectable (when the staining is now done at 4 C), demonstrating a rapid and selective loss of tegument-associated host proteins (Kemp *et al.*, 1980). The shedding was blocked by sodium fluoride, 2-deoxy-D-glucose and cytochalasin B treatment, suggesting that the process has some dependence on glucose uptake and metabolism (Kemp *et al.*, 1980). In other experiments, in the absence of metabolic inhibitors, surface immunoglobulin was lost within 2.5 hours when the parasites were incubated at 37 C (Kemp *et al.*, 1977). When complexed to their antigens, the surface immunoglobulins were lost within 10 minutes (Kemp *et al.*, 1977). These analyses strongly suggest that schistosomes have an ability to rapidly get rid of complexes built up on the external membrane (Torpier *et al.*, 1979b). It is of course possible, if the membranocalyx is fluid and lacks structural proteins such as tetraspanins, that host proteins move laterally in the bilayer to prominences where they are shed, in a manner analogous to the capping seen in mammalian cells.

In contrast, substantially slower turnover rates for the membranocalyx have also been reported. The rate of loss of labeled phosphatidylethanolamine associated with the schistosomula surface is given at a $t_{1/2}$ of ~12 hours (Caulfield *et al.*, 1991), and labeled LDL remained bound on the surface of schistosomula for up to 36 hours in culture (Bennett and Caulfield, 1991). In a second study, the host erythrocyte glycolipids present on the tegument surface were used as a marker to measure membranocalyx turnover (Saunders *et al.*, 1987). The amount of host antigen per worm was determined in an immunoradiometric assay, using a rabbit antibody to mouse erythrocyte ghosts. Antibody bound to the worm was detected with

donkey anti-rabbit IgG-biotin followed by 125 I-streptavidin. Experiments where worms were given a 30-minute pulse with cationized ferritin and then chase incubation lost half their host antigen in 7.4 hours. Continuous incubation with antiserum (or normal rabbit serum) did result in a slow loss corresponding to a mean half-life for host antigen *in vitro* of 45 hours. Surprisingly, giving worms a 30-minute pulse of the rabbit antiserum and then a chase incubation for 21 hours did not change the amount of host antigen detectable. These data suggest a very slow turnover of acquired erythrocyte glycans and possibly the entire membranocalyx. The findings were confirmed by worm transfer experiments where culture conditions are not an issue. Worms recovered from mice 28 days after infection were surgically transferred to the portal vein of Syrian hamsters, recovered again from the recipients between one and seven days after transfer and assayed for the presence of mouse erythrocyte glycolipids. There was a linear rate of loss *in vivo* with a half-life of 5.4 days. These data appear to rule out rapid, wholesale membranocalyx turnover as part of the immune evasion strategy, emphasizing its barrier function instead.

In other work, adult worms were incubated in normal human serum and then FITC-labeled anti-C3c was added. This gives rise to strong fluorescence over the entire parasite surface that, remarkably, persists for up to two weeks at 37 C *in vitro* (Ruppel and McLaren, 1986). On this basis, adult schistosomes would seem to be able to survive *in vitro* in the absence of rapid and general turnover of their membranocalyx (Ruppel and McLaren, 1986). Moreover, examination of these parasites by electron microscopy reveals little or no tegumental damage. Loss of fluorescence was noted consistently only at the anterior of the parasite, including the suckers, indicating that membrane turnover can occur at different rates on different parts of the body. Regional differences in membrane dynamics have previously been described (Johnson *et al.*, 1982).

In summary, the rate of membranocalyx turnover appears to vary depending on conditions in the external environment of the worm. In addition, regional differences in turnover are likely. The rates obtained with cationized ferritin appear exceptionally rapid and should perhaps be viewed as the maximum possible rate of replacement. Experiments involving host proteins, but not erythrocyte glycolipids,

also point to rapid shedding. In contrast, both *in vitro* and *in vivo* estimates for membranocalyx turnover using acquired glycolipid constituents as the marker suggest a very slow rate.

We have already alluded to the difficulty of demonstrating plasma membrane turnover because of the barrier to its labeling with probes presented by the membranocalyx. It is axiomatic that for every multilaminate vesicle that fuses with the surface of the tegument in adult worms, an equal amount of membrane must be removed if the surface area is not to increase progressively. The one piece of evidence that plasma membrane internalization actually occurs is provided by a study using a fluorescent styryl dye, FM 1-43 (Ribeiro *et al.*, 1998). Small vesicles formed after 10-minute incubation, a process blocked by incubation at 4 °C. The process is also inhibited with Primaquine, a disruptor of vesicle recycling in mammalian cells. Inhibition of tyrosine kinase signaling pathways with genistein also had some effect on vesicle formation (Ribeiro *et al.*, 1998). An investigation of this experimental system using confocal microscopy might reveal further details of the internalization, potentially of the plasma membrane back to the tegumental cytons, and into the endosomal compartment.

8. PRAZICQUANTEL AND THE TEGUMENT SURFACE

PZQ, (2-cyclohexylcarbonyl)-1,2,3,6,7,11b-hexa-hydro-2*H*-pyrazino [2,1*a*]isoquinolin-4-one, is the drug of choice for the chemotherapy of human schistosomiasis. At suboptimal doses it appears to require a functioning immune response to achieve its worm-killing potential. Thus, the efficacy of PZQ is significantly diminished in B-cell depleted mice (Brindley and Sher, 1987). Passive transfer of sera from infected animals reconstitutes PZQ's efficacy, demonstrating the synergy between the drug and the host's humoral immune response (Brindley and Sher, 1987; Doenhoff *et al.*, 1988). The effect of the drug is to change the stability of the tegumental membranes (Andrews, 1981; William *et al.*, 2001). Such changes at the worm surface increase exposure of parasite-specific antigens. The detection of IgG and IgM antibodies by immunofluorescence on the surface of adult worms recovered from intact mice, as early as one hour after

administration of the drug *in vivo* (Brindley and Sher, 1987) confirms the immunogenic nature of parasite surface molecules. Under normal circumstances, anti-tegumental antibodies must be circulating in the plasma of infected animals but unable to bind in sufficient quantity to damage an intact, unimpaired parasite. PZQ treatment exposes antigen to which pre-existing antibodies bind, to form a focus of immune mediated and irreversible damage to the tegument.

Some of the surface targets of lethal antibody have been identified. They include a 27-kDa esterase (Doenhoff *et al.*, 1988), alkaline phosphatase (Fallon *et al.*, 1994) and a 200-kDa tubercle glycoprotein, possibly Sm200 (see below) (Brindley *et al.*, 1989). Antibody against these molecules alone can act synergistically with PZQ to promote worm elimination (Doenhoff *et al.*, 1988; Brindley *et al.*, 1989; Fallon *et al.*, 1994). For instance, anti-alkaline phosphatase antiserum when administered to PZQ-treated mice, significantly and preferentially enhanced the death of female worms (Fallon *et al.*, 1994), whereas administration in the absence of PZQ treatment had no effect on the parasites. This preferential binding of anti-alkaline phosphatase antibody to the female tegument is in keeping with the reported distribution of alkaline phosphatase in all areas of the female tegument (Ninno-Smith and Standen, 1963).

Likewise, the 200-kDa protein described earlier (Section 2.6.4) is a target of antibodies that act in synergy with PZQ and its release from the surface with PIPLC is about five times more extensive in the presence of PZQ (Sauma *et al.*, 1991). A polyclonal antiserum against the *S. mansoni* 200-kDa protein cross reacts with 200-kDa proteins of two other schistosome species—*S. haematobium* and *S. japonicum* (Tanaka *et al.*, 1993). The 200-kDa protein of *S. haematobium*, like its counterpart in *S. mansoni*, was exposed following PZQ treatment. In contrast, treatment with PZQ did not result in increased exposure of the 200-kDa protein of *S. japonicum*, confirming surface biochemical differences between schistosome species (Tanaka *et al.*, 1993).

Interestingly, an anti-paramyosin antibody did not react with the surface of PZQ-treated schistosomes (Brindley *et al.*, 1989). Other schistosome molecules listed earlier (Tables 1 and 2) as being at the host-parasite interface (e.g. SGTP4, ATP diphosphohydrolase, phosphodiesterase, Sm29) would represent further candidates against

which antibody with PZQ may synergistically act. PZQ-induced damage of the parasite's tegument exposes surface spines, as determined by immunofluorescence using an anti-actin antibody (Linder and Thors, 1992). Spine disintegration is seen *in vivo* (Linder and Thors, 1992).

While PZQ treatment exposes parasite antigen, there is no demonstrable effect on acquired host molecules (Harnett and Kusel, 1986). Anti-host erythrocyte serum reacts with the parasite surface to a similar degree before or following PZQ treatment (Harnett and Kusel, 1986; Brindley and Sher, 1987). Thus no loss in host tegumental antigens can be detected on worms from PZQ-treated mice over a period of days (Brindley and Sher, 1987).

9. SUMMATION

In this review, we have explored the biochemical nature of the tegument surface of intra-mammalian schistosomes. We have used a conceptual model of that surface as a plasma membrane overlain by a secreted membranocalyx to provide a context for the many papers published since the tegument ultrastructure was first described nearly 40 years ago. It is clear to us that much remains to be discovered before we can explain the mechanism(s) of immune evasion that schistosomes deploy at the tegument surface to enable them to survive in the host bloodstream for many years. Not least is the precise location of proteins and glycans in the plasma membrane, the membranocalyx and the space in between.

Paradoxically, in view of its robustness *in vivo*, the tegument is easily damaged by handling *in vitro*, and this creates a major obstacle for interpretation of the literature. We believe that this fragility accounts for many of the reports of cytosolic and cytoskeletal proteins exposed on the tegument surface. Indeed, in only a few instances do the newer proteomic analyses corroborate the earlier reports (Tables 1 and 2). In addition, we make a distinction regarding the composition of the surface in newly transformed schistosomula versus developing and adult worms. During transformation, the old cercarial plasma membrane is shed and replaced by preformed packages of material from the cell bodies. It is during this period that, we propose,

numerous 'internal' proteins can be detected at the surface, but their exposure is transient. Membranocalyx formation is rapid and from 24 hours onwards it provides a barrier to effector molecules and cells of the immune system.

The proteomic analyses reveal a range of enzymes, including phosphohydrolases and esterases, in the surface complex (Table 2). In addition, transporters of nutrients, inorganic ions and water have been identified in the tegument membrane preparations. A number of tetraspanins have been detected that probably provide a structural framework for the other membrane proteins, perhaps segregated into distinct domains that coincide with morphological features such as surface pits. Conversely, secreted proteins appear to be relatively scarce, with convincing evidence for only two, Sm13 and Sm29, external to the plasma membrane. It is notable that few receptors have been identified in the surface membrane preparations by MS. The absence of conventional receptors for the Fc region of immunoglobulin and for complement, both reported at the tegument surface, is striking. However, a number of proteins with no known homology have been identified, which could represent novel but elusive versions of such receptors. One incentive for the characterization of the surface proteins is that the most exposed might be accessible to host immune effectors and would make good vaccine candidates; selected molecules are in the process of being tested as such.

In terms of host proteins at the tegument surface, strong evidence exists for the presence of bound immunoglobulin, whether to parasite antigens as part of an active, anti-tegument response or via Fc receptors is unresolved (Tables 3 and 4). The identification of a degraded Complement C3 fragment suggests the former option, as does the presence of Crry, a murine Complement regulatory protein. Numerous other host proteins, both GPI-anchored and single membrane spanning, have also been detected by compositional analysis of a surface membrane preparation (Table 4). The importance of host glycosphingolipids at the parasite surface is unknown. Do they provide a barrier to host immune effectors or are they more simply passively absorbed?

It is worth emphasizing that the host-interactive schistosome surface is not a molecularly uniform structure. Analysis of lipid analogs

applied to the parasites shows that the tegumental membranes are differentiated into domains of differing composition. The diffusion rates of different lipids in these membranes can vary substantially suggesting that lipids may be sequestered into micro-domains that may have specialized functions such as nutrient uptake or signaling. The molecular composition of the microdomains and the functions of the molecules comprising them are largely unknown. There are still unanswered questions about plasma membrane and membranocalyx turnover. The original observations that suggested a rapid sloughing of the latter now appear to be a product of the experimental system, and later observations suggest a much slower process. Nevertheless, it seems clear that surface material is shed into the bloodstream, and that it contains macromolecules of parasite origin that could be antigenic. That the host does not, or cannot attack these parasite molecules means they are either poorly immunogenic or well shielded from antibodies, possibly by the covering of host molecules. We feel that the most plausible mechanism of membranocalyx function is to act as a physical and relatively inert barrier between the host environment and the parasite. However, the barrier cannot be absolute because of the need for nutrients, ions and enzyme substrates (all small molecules) to cross it and reach the proteins of the underlying plasma membrane. For this to happen, it is necessary to postulate the existence of pores, or at the very least discontinuities in the membranocalyx. The question of plasma membrane turnover also remains to be settled, but the likelihood is that its constituents are not turned over into the environment. Instead, they are likely recycled back to endosomal compartments in the cell bodies, as is the norm for secretory cells. It just so happens that the configuration of the schistosome tegument makes this technically difficult to demonstrate.

REFERENCES

- Abath, F.G. and Werkhauser, R.C. (1996). The tegument of *Schistosoma mansoni*: functional and immunological features. *Parasite Immunology* **18**, 15–20.

- Abath, F.G., Hirst, E.M., Hagan, P. and Simpson, A.J. (1999). Ultrastructural localization of Sm15 and Sm25, two major tegumental adult worm antigens of *Schistosoma mansoni*. *Parasite* **6**, 243–247.
- Ali, P.O., Jeffs, S.A., Meadows, H.M., Hollyer, T., Owen, C.A., Abath, F.G., Allen, R., Hackett, F., Smithers, S.R. and Simpson, A.J. (1991). Structure of Sm25, an antigenic integral membrane glycoprotein of adult *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **45**, 215–222.
- Allan, D., Payares, G. and Evans, W.H. (1987). The phospholipid and fatty acid composition of *Schistosoma mansoni* and of its purified tegumental membranes. *Molecular and Biochemical Parasitology* **23**, 123–128.
- Anderson, R.G. (1998). The caveolae membrane system. *Annual Review of Biochemistry* **67**, 199–225.
- Andrews, P. (1981). A summary of the efficacy of praziquantel against schistosomes in animal experiments and notes on its mode of action. *Arzneimittel-Forschung* **31**, 538–541.
- Argiro, L., Henri, S., Dessein, H., Dessein, A.J. and Bourgois, A. (1999). Induction of a protective immunity against *Schistosoma mansoni* with ovalbumin-coupled Sm37-5 coadsorbed with granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-12 on alum. *Vaccine* **17**, 13–18.
- Argiro, L., Henri, S., Dessein, H., Kouriba, B., Dessein, A.J. and Bourgois, A. (2000a). Induction of a protection against *S. mansoni* with a MAP containing epitopes of Sm37-GAPDH and Sm10-DLC. Effect of coadsorption with GM-CSF on alum. *Vaccine* **18**, 2033–2038.
- Argiro, L.L., Kohlstadt, S.S., Henri, S.S., Dessein, H.H., Matabiau, V.V., Paris, P.P., Bourgois, A.A. and Dessein, A.J. (2000b). Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. *Vaccine* **18**, 2039–2048.
- Arnon, R., Silman, I. and Tarrab-Hazdai, R. (1999). Acetylcholinesterase of *Schistosoma mansoni* functional correlates. Contributed in honor of Professor Hans Neurath's 90th birthday. *Protein Science* **8**, 2553–2561.
- Asch, H.L. and Read, C.P. (1975). Membrane transport in *Schistosoma mansoni*: transport of amino acids by adult males. *Experimental Parasitology* **38**, 123–135.
- Balloul, J.M., Grzych, J.M., Pierce, R.J. and Capron, A. (1987). A purified 28,000 dalton protein from *Schistosoma mansoni* adult worms protects rats and mice against experimental schistosomiasis. *Journal of Immunology* **138**, 3448–3453.
- Beall, M.J. and Pearce, E.J. (2001). Human transforming growth factor-beta activates a receptor serine threonine kinase from the intravascular parasite *Schistosoma mansoni*. *Journal of Biological Chemistry* **276**, 31613–31619.
- Beall, M.J., McGonigle, S. and Pearce, E.J. (2000). Functional conservation of *Schistosoma mansoni* Smads in TGF-beta signaling. *Molecular and Biochemical Parasitology* **111**, 131–142.

- Bennett, M.W. and Caulfield, J.P. (1991). Specific binding of human low-density lipoprotein to the surface of schistosomula of *Schistosoma mansoni* and ingestion by the parasite. *American Journal of Pathology* **138**, 1173–1182.
- Bennett, J.L. and Seed, J.L. (1977). Characterization and isolation of concanavalin: a binding sites from the epidermis of *S. mansoni*. *Journal of Parasitology* **63**, 250–258.
- Bickle, Q.D. and Ford, M.J. (1982). Studies on the surface antigenicity and susceptibility to antibody-dependent killing of developing schistosomula using sera from chronically infected mice and mice vaccinated with irradiated cercariae. *Journal of Immunology* **128**, 2101–2106.
- Bogitsh, B.J. and Krupa, P.L. (1971). *Schistosoma mansoni* and *Haematoloechus medioplexus*: nucleosidediphosphatase localization in tegument. *Experimental Parasitology* **30**, 418–425.
- Botti, S.A., Felder, C.E., Sussman, J.L. and Silman, I. (1998). Electrotactins: a class of adhesion proteins with conserved electrostatic and structural motifs. *Protein Engineering* **11**, 415–420.
- Boyer, M.H. and Ketchum, D.G. (1976). The host antigen phenomenon in experimental murine schistosomiasis. II. Failure to demonstrate destruction of parasites transferred from hamsters to mice. *Journal of Immunology* **116**, 1093–1095.
- Boyer, M.H., Ketchum, D.G. and Palmer, P.D. (1976). The host antigen phenomenon in experimental murine schistosomiasis: the transfer of 3-week old *Schistosoma mansoni* between two inbred strains of mice. *International Journal for Parasitology* **6**, 235–238.
- Braschi, S. and Wilson, R.A. (2006). Proteins exposed at the adult schistosome surface revealed by biotinylation. *Molecular and Cellular Proteomics* **5**, 347–356.
- Braschi, S., Curwen, R.S., Ashton, P.D., Verjovski-Almeida, S. and Wilson, R.A. (2006). The tegument surface membrane of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction. *Proteomics* **6**, 1471–1482.
- Brindley, P.J. and Sher, A. (1987). The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. *Journal of Immunology* **139**, 215–220.
- Brindley, P.J., Strand, M., Norden, A.P. and Sher, A. (1989). Role of host antibody in the chemotherapeutic action of praziquantel against *Schistosoma mansoni*: identification of target antigens. *Molecular and Biochemical Parasitology* **34**, 99–108.
- Brophy, P.M. and Barrett, J. (1990). Glutathione transferase in helminths. *Parasitology* **100**(Pt 2), 345–349.
- Brouwers, J.F., Skelly, P.J., Van Golde, L.M. and Tielens, A.G. (1999). Studies on phospholipid turnover argue against sloughing of tegumental membranes in adult *Schistosoma mansoni*. *Parasitology* **119**, 287–294.

- Butterworth, A.E., Vadas, M.A., Martz, E. and Sher, A. (1979). Cytolytic T lymphocytes recognize alloantigens on schistosomula of *Schistosoma mansoni*, but fail to induce damage. *Journal of Immunology* **122**, 1314–1321.
- Camacho, M. and Agnew, A. (1995). Schistosoma: rate of glucose import is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. *Experimental Parasitology* **81**, 584–591.
- Camacho, M., Alsford, S. and Agnew, A. (1996). Molecular forms of tegumental and muscle acetylcholinesterases of *Schistosoma*. *Parasitology* **112**(Pt 2), 199–204.
- Camacho, M., Tarrab-Hazdai, R., Espinoza, B., Arnon, R. and Agnew, A. (1994). The amount of acetylcholinesterase on the parasite surface reflects the differential sensitivity of schistosome species to metrifonate. *Parasitology* **108**(Pt 2), 153–160.
- Camacho, M., Alsford, S., Jones, A. and Agnew, A. (1995). Nicotinic acetylcholine receptors on the surface of the blood fluke *Schistosoma*. *Molecular and Biochemical Parasitology* **71**, 127–134.
- Carlisle, S., Weisberg, L.S. and Bentley, A.G. (1983). *Schistosoma mansoni*: morphologic changes induced by maintenance *in vitro*. *Journal of Parasitology* **69**, 319–334.
- Caulfield, J.P., Korman, G., Butterworth, A.E., Hogan, M. and David, J.R. (1980a). The adherence of human neutrophils and eosinophils to schistosomula: evidence for membrane fusion between cells and parasites. *Journal of Cell Biology* **86**, 46–63.
- Caulfield, J.P., Korman, G., Butterworth, A.E., Hogan, M. and David, J.R. (1980b). Partial and complete detachment of neutrophils and eosinophils from schistosomula: evidence for the establishment of continuity between a fused and normal parasite membrane. *Journal of Cell Biology* **86**, 64–76.
- Caulfield, J.P., Chiang, C.P., Yacono, P.W., Smith, L.A. and Golan, D.E. (1991). Low density lipoproteins bound to *Schistosoma mansoni* do not alter the rapid lateral diffusion or shedding of lipids in the outer surface membrane. *Journal of Cell Science* **99**, 167–173.
- Cesari, I.M. (1974). *Schistosoma mansoni*: distribution and characteristics of alkaline and acid phosphatase. *Experimental Parasitology* **36**, 405–414.
- Cesari, I.M., Simpson, A.J. and Evans, W.H. (1981). Properties of a series of tegumental membrane-bound phosphohydrolase activities of *Schistosoma mansoni*. *Biochemical Journal* **198**, 467–473.
- Chiang, C.P. and Caulfield, J.P. (1989). Human lipoprotein binding to schistosomula of *Schistosoma mansoni*. Displacement by polyanions, parasite antigen masking, and persistence in young larvae. *American Journal of Pathology* **135**, 1015–1024.
- Chirathaworn, C., Kohlmeier, J.E., Tibbetts, S.A., Rumsey, L.M., Chan, M.A. and Benedict, S.H. (2002). Stimulation through intercellular adhesion molecule-1 provides a second signal for T cell activation. *Journal of Immunology* **168**, 5530–5537.

- Clegg, J.A., Smithers, S.R. and Terry, R.J. (1970). "Host" antigens associated with schistosomes: observations on their attachment and their nature. *Parasitology* **61**, 87–94.
- Clegg, J.A., Smithers, S.R. and Terry, R.J. (1971a). Acquisition of human antigens by *Schistosoma mansoni* during cultivation *in vitro*. *Nature* **232**, 653–654.
- Clegg, J.A., Smithers, S.R. and Terry, R.J. (1971b). Concomitant immunity and host antigens associated with schistosomiasis. *International Journal for Parasitology* **1**, 43–49.
- Cohen, C., Reinhardt, B., Castellani, L., Norton, P. and Stirewalt, M. (1982). Schistosome surface spines are "crystals" of actin. *Journal of Cell Biology* **95**, 987–988.
- Cordeiro, M.N. and Gazzinelli, G. (1979). *Schistosoma mansoni*: resolution and molecular weight estimates of tegument glycoproteins by polyacrylamide gel electrophoresis. *Experimental Parasitology* **48**, 337–344.
- Crabtree, J.E. and Wilson, R.A. (1986). *Schistosoma mansoni*: an ultrastructural examination of pulmonary migration. *Parasitology* **92**(Pt 2), 343–354.
- Cutts, L. and Wilson, R.A. (1997). The protein antigens secreted *in vivo* by adult male *Schistosoma mansoni*. *Parasitology* **114**(Pt 3), 245–255.
- Da'dara, A., Tsai, M.H., Tao, L.F., Marx, K.A., Shoemaker, C.B., Harn, D.A. and Skelly, P.J. (2001a). *Schistosoma mansoni*: molecular characterization of a tegumental Ca-ATPase (SMA3). *Experimental Parasitology* **98**, 215–222.
- Da'dara, A.A., Skelly, P.J., Wang, M.M. and Harn, D.A. (2001b). Immunization with plasmid DNA encoding the integral membrane protein, Sm23, elicits a protective immune response against schistosome infection in mice. *Vaccine* **20**, 359–369.
- Da'dara, A.A., Skelly, P.J., Fatakda, M., Visocatti, S., Eriksson, E. and Harn, D.A. (2002). Comparative efficacy of the *Schistosoma mansoni* nucleic acid vaccine, Sm23, following microseeding or gene gun delivery. *Parasite Immunology* **24**, 179–187.
- Dalton, J.P. and Strand, M. (1987). *Schistosoma mansoni* polypeptides immunogenic in mice vaccinated with radiation-attenuated cercariae. *Journal of Immunology* **139**, 2474–2481.
- Damian, R.T. (1967). Common antigens between adult *Schistosoma mansoni* and the laboratory mouse. *Journal of Parasitology* **53**, 60–64.
- Damian, R.T., Greene, N.D. and Hubbard, W.J. (1973). Occurrence of mouse 2-macroglobulin antigenic determinants on *Schistosoma mansoni* adults, with evidence on their nature. *Journal of Parasitology* **59**, 64–73.
- Dave, K.R., Syal, A.R. and Katyare, S.S. (2000). Tissue cholinesterases. A comparative study of their kinetic properties. *Z Naturforsch C* **55**, 100–108.
- Davies, S.J. and Pearce, E.J. (1995). Surface-associated serine-threonine kinase in *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **70**, 33–44.

- Davies, S.J., Shoemaker, C.B. and Pearce, E.J. (1998). A divergent member of the transforming growth factor beta receptor family from *Schistosoma mansoni* is expressed on the parasite surface membrane. *Journal of Biological Chemistry* **273**, 11234–11240.
- Dean, D.A. (1974). *Schistosoma mansoni*: adsorption of human blood group A and B antigens by schistosomula. *Journal of Parasitology* **60**, 260–263.
- Dean, D.A. (1977). Decreased binding of cytotoxic antibody by developing *Schistosoma mansoni*. Evidence for a surface change independent of host antigen adsorption and membrane turnover. *Journal of Parasitology* **63**, 418–426.
- Dean, D.A. and Sell, K.W. (1972). Surface antigens on *Schistosoma mansoni*. II. Adsorption of a Forssman-like host antigen by schistosomula. *Clinical Experimental Immunology* **12**, 525–540.
- Deng, J., Gold, D., LoVerde, P.T. and Fishelson, Z. (2003). Inhibition of the complement membrane attack complex by *Schistosoma mansoni* paramyosin. *Infection and Immunity* **71**, 6402–6410.
- Dessein, A., Samuelson, J.C., Butterworth, A.E., Hogan, M., Sherry, B.A., Vadas, M.A. and David, J.R. (1981). Immune evasion by *Schistosoma mansoni*: loss of susceptibility to antibody or complement-dependent eosinophil attack by schistosomula cultured in medium free of macromolecules. *Parasitology* **82**, 357–374.
- Dessein, A.J., Begley, M., Demeure, C., Caillol, D., Fueri, J., dos Reis, M.G., Andrade, Z.A., Prata, A. and Bina, J.C. (1988). Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kDa larval surface antigen. *Journal of Immunology* **140**, 2727–2736.
- Dissous, C. and Capron, A. (1981). Isolation and characterization of surface antigens from *Schistosoma mansoni* schistosomula. *Molecular and Biochemical Parasitology* **3**, 215–225.
- Doenhoff, M.J., Modha, J. and Lambertucci, J.R. (1988). Anti-schistosome chemotherapy enhanced by antibodies specific for a parasite esterase. *Immunology* **65**, 507–510.
- Dorsey, C. and Cousin, C. (1995). Cyton II: a subtegumental cell type in the cercaria of *Schistosoma mansoni*. *Journal of Morphology* **224**, 233–240.
- Dusanic, D.G. (1959). Histochemical observations of alkaline phosphatase in *Schistosoma mansoni*. *Journal of Infectious Diseases* **105**, 1–8.
- Espinoza, B., Tarrab-Hazdai, R., Silman, I. and Arnon, R. (1988). Acetylcholinesterase in *Schistosoma mansoni* is anchored to the membrane via covalently attached phosphatidylinositol. *Molecular and Biochemical Parasitology* **29**, 171–179.
- Espinoza, B., Silman, I., Arnon, R. and Tarrab-Hazdai, R. (1991a). Phosphatidylinositol-specific phospholipase C induces biosynthesis of acetylcholinesterase via diacylglycerol in *Schistosoma mansoni*. *European Journal of Biochemistry* **195**, 863–870.

- Espinoza, B., Tarrab-Hazdai, R., Himmeloch, S. and Arnon, R. (1991b). Acetylcholinesterase from *Schistosoma mansoni*: immunological characterization. *Immunology Letters* **28**, 167–174.
- Espinoza, B., Parizade, M., Ortega, E., Tarrab-Hazdai, R., Zilberg, D. and Arnon, R. (1995). Monoclonal antibodies against acetylcholinesterase of *Schistosoma mansoni*: production and characterization. *Hybridoma* **14**, 577–586.
- Fallon, P.G., Smith, P., Nicholls, T., Modha, J. and Doenhoff, M.J. (1994). Praziquantel-induced exposure of *Schistosoma mansoni* alkaline phosphatase: drug-antibody synergy which acts preferentially against female worms. *Parasite Immunology* **16**, 529–535.
- Ferguson, M.A.J. (1994). What can GPI do for you? *Parasitology Today* **10**, 48–52.
- Foley, M., MacGregor, A.N., Kusel, J.R., Garland, P.B., Downie, T. and Moore, I. (1986). The lateral diffusion of lipid probes in the surface membrane of *Schistosoma mansoni*. *Journal of Cell Biology* **103**, 807–818.
- Fripp, P.J. (1967a). Histochemical localization of esterase activity in Schistosomes. *Experimental Parasitology* **21**, 380–390.
- Fripp, P.J. (1967b). The histochemical localization of leucine aminopeptidase activity in *Schistosoma rodhaini*. *Comparative Biochemistry and Physiology B* **20**, 307–309.
- Fripp, P.J. (1967c). The sites of (1-14C) glucose assimilation in *Schistosoma haematobium*. *Comparative Biochemistry and Physiology* **23**, 893–898.
- Gearner, G.W. and Kemp, W.M. (1994). Electrophoretic and serological analysis of host antigens associated with the adult *Schistosoma mansoni* tegument. *Journal of Parasitology* **80**, 275–283.
- Ghendler, Y., Arnon, R. and Fishelson, Z. (1994). *Schistosoma mansoni*: isolation and characterization of Smpi56, a novel serine protease inhibitor. *Experimental Parasitology* **78**, 121–131.
- Ghendler, Y., Parizade, M., Arnon, R., McKerrow, J.H. and Fishelson, Z. (1996). *Schistosoma mansoni*: evidence for a 28-kDa membrane-anchored protease on schistosomula. *Experimental Parasitology* **83**, 73–82.
- Gitter, B.D. and Damian, R.T. (1982). Murine alloantigen acquisition by schistosomula of *Schistosoma mansoni*: further evidence for the presence of K, D, and I region gene products on the tegumental surface. *Parasite Immunology* **4**, 383–393.
- Gitter, B.D., McCormick, S.L. and Damian, R.T. (1982). Murine alloantigen acquisition by *Schistosoma mansoni*: presence of H-2 K determinants on adult worms and failure of allogeneic lymphocytes to recognize acquired MHC gene products on schistosomula. *Journal of Parasitology* **68**, 513–518.
- Gobert, G.N., Stenzel, D.J., Jones, M.K., Allen, D.E. and McManus, D.P. (1997). *Schistosoma japonicum*: Immunologyocalization of paramyosin during development. *Parasitology* **114**(Pt 1), 45–52.

- Goldring, O.L., Clegg, J.A., Smithers, S.R. and Terry, R.J. (1976). Acquisition of human blood group antigens by *Schistosoma mansoni*. *Clinical Experimental Immunology* **26**, 181–187.
- Goldring, O.L., Kusel, J.R. and Smithers, S.R. (1977a). *Schistosoma mansoni*: origin *in vitro* of host-like surface antigens. *Experimental Parasitology* **43**, 82–93.
- Goldring, O.L., Sher, A., Smithers, S.R. and McLaren, D.J. (1977b). Host antigens and parasite antigens of murine *Schistosoma mansoni*. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **71**, 144–148.
- Goudot-Crozel, V., Caillol, D., Djabali, M. and Dessein, A.J. (1989). The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kD glyceraldehyde-3P-dehydrogenase. *Journal of Experimental Medicine* **170**, 2065–2080.
- Hall, T.M., Joseph, G.T. and Strand, M. (1995). *Schistosoma mansoni*: molecular cloning and sequencing of the 200-kDa chemotherapeutic target antigen. *Experimental Parasitology* **80**, 242–249.
- Halton, D.W. (1967). Studies on phosphatase activity in trematoda. *Journal of Parasitology* **53**, 46–54.
- Harn, D.A., Mitsuyama, M., Huguenel, E.D. and David, J.R. (1985). *Schistosoma mansoni*: detection by monoclonal antibody of a 22,000-dalton surface membrane antigen which may be blocked by host molecules on lung stage parasites. *Journal of Immunology* **135**, 2115–2120.
- Harn, D.A., Gu, W., Oligino, L.D., Mitsuyama, M., Gebremichael, A. and Richter, D. (1992). A protective monoclonal antibody specifically recognizes and alters the catalytic activity of schistosome triose-phosphate isomerase. *Journal of Immunology* **148**, 562–567.
- Harnett, W. and Kusel, J.R. (1986). Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. *Parasitology* **93**(Pt 2), 401–405.
- Harnett, W., Kusel, J.R. and Barrowman, M.M. (1985). The use of aldehydes to show a relationship between host and parasite antigens at the surface of adult male *Schistosoma mansoni*. *Parasite Immunology* **7**, 415–428.
- Havercroft, J.C. and Smith, A.L. (1993). Localization of the A1.12.9 antigen family to the neurones, putative sensory receptors and tegument of *Schistosoma mansoni*. *Parasite Immunology* **15**, 361–371.
- Hernandez, M.G., Hafalla, J.C., Acosta, L.P., Aligui, F.F., Aligui, G.D., Ramirez, B.L., Dunne, D.W. and Santiago, M.L. (1999). Paramyosin is a major target of the human IgA response against *Schistosoma japonicum*. *Parasite Immunology* **21**, 641–647.
- Hockley, D.J. (1973). Ultrastructure of the tegument of *Schistosoma*. *Advances in Parasitology* **11**, 233–305.
- Hockley, D.J. and McLaren, D.J. (1973). *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. *International Journal for Parasitology* **3**, 13–25.

- Hockley, D.J., McLaren, D.J., Ward, B.J. and Nermut, M.V. (1975). A freeze-fracture study of the tegumental membrane of *Schistosoma mansoni* (Platyhelminthes: Trematoda). *Tissue Cell* **7**, 485–496.
- Holy, J.M., O'Leary, K.A., Oaks, J.A. and Tracy, J.W. (1989). Immunocytochemical localization of the major glutathione S-transferases in adult *Schistosoma mansoni*. *Journal of Parasitology* **75**, 181–190.
- Horta, M.F., Ramalho-Pinto, F.J. and Fatima, M. (1991). Role of human decay-accelerating factor in the evasion of *Schistosoma mansoni* from the complement-mediated killing *in vitro*. *Journal of Experimental Medicine* **174**, 1399–1406.
- Imase, A., Kobayashi, K., Ohmae, H., Matsuda, H. and Iwamura, Y. (2001). Horizontal and vertical transmission of mouse class I MHC sequence in *Schistosoma mansoni*. *Parasitology* **123**, 163–168.
- Inal, J.M. (1999). Schistosoma TOR (trispinning orphan receptor). A novel, antigenic surface receptor of the blood-dwelling, Schistosoma parasite. *Biochimica et Biophysica Acta* **1445**, 283–298.
- Inal, J.M. and Schifferli, J.A. (2002). Complement C2 receptor inhibitor trispinning and the beta-chain of C4 share a binding site for complement C2. *Journal of Immunology* **168**, 5213–5221.
- Inal, J.M. and Sim, R.B. (2000). A Schistosoma protein, Sh-TOR, is a novel inhibitor of complement which binds human C2. *FEBS Letters* **470**, 131–134.
- Inatomi, S., Tongu, Y., Sakumoto, D., Suguri, S. and Itano, K. (1969). The ultrastructure of helminth (3) the body wall of *Schistosoma japonicum*. *Japanese Journal of Parasitology* **18**, 174–181.
- Jiang, J., Skelly, P.J., Shoemaker, C.B. and Caulfield, J.P. (1996). *Schistosoma mansoni*: the glucose transport protein SGTP4 is present in tegumental multilamellar bodies, discoid bodies, and the surface lipid bilayers. *Experimental Parasitology* **82**, 201–210.
- Johnson, P., Garland, P.B., Campbell, P. and Kusel, J.R. (1982). Changes in the properties of the surface membrane of *Schistosoma mansoni* during growth as measured by fluorescence recovery after photobleaching. *FEBS Letters* **141**, 132–135.
- Jones, A.K., Bentley, G.N., Oliveros Parra, W.G. and Agnew, A. (2002). Molecular characterization of an acetylcholinesterase implicated in the regulation of glucose scavenging by the parasite *Schistosoma*. *FASEB Journal* **16**, 441–443.
- Kabil, S.M. (1976). Host complement in the schistosomal tegument. *Journal of Tropical Medicine and Hygiene* **79**, 205–206.
- Kemp, W.M., Damian, R.T. and Greene, N.D. (1976a). Immunocytochemical localization of IgG on adult *Schistosoma mansoni* tegumental surfaces. *Journal of Parasitology* **62**, 830–832.
- Kemp, W.M., Damian, R.T., Greene, N.D. and Lushbaugh, W.B. (1976b). Immunocytochemical localization of mouse alpha 2-macroglobulinlike

- antigenic determinants on *Schistosoma mansoni* adults. *Journal of Parasitology* **62**, 413–419.
- Kemp, W.M., Merritt, S.C., Bogucki, M.S., Rosier, J.G. and Seed, J.R. (1977). Evidence for adsorption of heterospecific host immunoglobulin on the tegument of *Schistosoma mansoni*. *Journal of Immunology* **119**, 1849–1854.
- Kemp, W.M., Merritt, S.C. and Rosier, J.G. (1978). *Schistosoma mansoni*: identification of immunoglobulins associated with the tegument of adult parasites from mice. *Experimental Parasitology* **45**, 81–87.
- Kemp, W.M., Brown, P.R., Merritt, S.C. and Miller, R.E. (1980). Tegument-associated antigen modulation by adult male *Schistosoma mansoni*. *Journal of Immunology* **124**, 806–811.
- Ko, A.L., Drager, U.C. and Harn, D.A. (1990). A *Schistosoma mansoni* epitope recognized by a protective monoclonal antibody is identical to the stage-specific embryonic antigen 1. *Proceedings of the National Academy of Sciences USA* **87**, 4159–4163.
- Koster, B. and Strand, M. (1994a). *Schistosoma mansoni*: immunology-localization of two different fucose-containing carbohydrate epitopes. *Parasitology* **108**(Pt 4), 433–446.
- Koster, B. and Strand, M. (1994b). *Schistosoma mansoni*: Sm23 is a transmembrane protein that also contains a glycosylphosphatidylinositol anchor. *Archives of Biochemistry and Biophysics* **310**, 108–117.
- Koster, B., Hall, M.R. and Strand, M. (1993). *Schistosoma mansoni*: immunoreactivity of human sera with the surface antigen Sm23. *Experimental Parasitology* **77**, 282–294.
- Kruger, F.J. and Wolmarans, C.T. (1990). Host cellular components adhering to the tegument of schistosomes from cattle, buffalo, hippopotamus and lechwe. *Onderstepoort Journal of Veterinary Research* **57**, 137–139.
- Kusel, J.R. and Gordon, J.F. (1989). Biophysical studies of the schistosome surface and their relevance to its properties under immune and drug attack. *Parasite Immunology* **11**, 431–451.
- Kusel, J.R. and Mackenzie, P.E. (1975). The measurement of the relative turnover rates of proteins of the surface membranes and other fractions of *Schistosoma mansoni* in culture. *Parasitology* **71**, 261–273.
- Kusel, J.R., Mackenzie, P.E. and McLaren, D.J. (1975). The release of membrane antigens into culture by adult *Schistosoma mansoni*. *Parasitology* **71**, 247–259.
- Laclette, J.P., Shoemaker, C.B., Richter, D., Arcos, L., Pante, N., Cohen, C., Bing, D. and Nicholson-Weller, A. (1992). Paramyosin inhibits complement C1. *Journal of Immunology* **148**, 124–128.
- Laclette, J.P., Skelly, P.J., Merchant, M.T. and Shoemaker, C.B. (1995). Aldehyde fixation dramatically alters the immunology localization pattern of paramyosin in platyhelminth parasites. *Experimental Parasitology* **81**, 140–143.

- Lee, D.L. (1996). Why do some nematode parasites of the alimentary tract secrete acetylcholinesterase? *International Journal for Parasitology* **26**, 499–508.
- Lee, K.W., Shalaby, K.A., Medhat, A.M., Shi, H., Yang, Q., Karim, A.M. and LoVerde, P.T. (1995). *Schistosoma mansoni*: characterization of the gene encoding Sm23, an integral membrane protein. *Experimental Parasitology* **80**, 155–158.
- Levi-Schaffer, F., Tarrab-Hazdai, R., Schryer, M.D., Arnon, R. and Smolarsky, M. (1984). Isolation and partial characterization of the tegumental outer membrane of schistosomula of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **13**, 283–300.
- Linder, E. and Hultdt, G. (1983). Antibody-independent binding and activation of complement by *Schistosoma mansoni* adult worms. *Parasite Immunology* **5**, 183–194.
- Linder, E. and Thors, C. (1992). *Schistosoma mansoni*: praziquantel-induced tegumental lesion exposes actin of surface spines and allows binding of actin depolymerizing factor, gelsolin. *Parasitology* **105**(Pt 1), 71–79.
- Loukas, A., Jones, M.K., King, L.T., Brindley, P.J. and McManus, D.P. (2001). Receptor for Fc on the surfaces of schistosomes. *Infection and Immunity* **69**, 3646–3651.
- Maecker, H.T., Todd, S.C. and Levy, S. (1997). The tetraspanin superfamily: molecular facilitators. *FASEB Journal* **11**, 428–442.
- Mansour, J.M., McCrossan, M.V., Bickle, Q.D. and Mansour, T.E. (2000). *Schistosoma mansoni* phosphofructokinase: immunologyocalization in the tegument and immunogenicity. *Parasitology* **120**(Pt 5), 501–511.
- Marikovsky, M., Parizade, M., Arnon, R. and Fishelson, Z. (1990). Complement regulation on the surface of cultured schistosomula and adult worms of *Schistosoma mansoni*. *European Journal of Immunology* **20**, 221–227.
- Marshall, I. (1987). Experimental chemotherapy. In: *The Biology of Schistosomes. From Genes to Latrines* (D. Rollinson and A.J.G. Simpson, eds), pp. 399–430. London: Academic Press.
- Massoulie, J., Anselmet, A., Bon, S., Krejci, E., Legay, C., Morel, N. and Simon, S. (1998). Acetylcholinesterase: C-terminal domains, molecular forms and functional localization. *Journal of Physiology* **92**, 183–190.
- Mastroberardino, L., Spindler, B., Pfeiffer, R., Skelly, P.J., Loffing, J., Shoemaker, C.B. and Verrey, F. (1998). Amino-acid transport by heterodimers of 4F2hc/CD98 and members of a permease family. *Nature* **395**, 288–291.
- Matsumoto, Y., Perry, G., Levine, R.J., Blanton, R., Mahmoud, A.A. and Aikawa, M. (1988). Paramyosin and actin in schistosomal teguments. *Nature* **333**, 76–78.
- McDiarmid, S.S. and Podesta, R.B. (1984). Identification of a sialic acid containing glycocalyx on the surface of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **10**, 33–43.

- McDiarmid, S.S., Podesta, R.B. and Rahman, S.M. (1982). Preparation and partial characterisation of a multilamellar body fraction from *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **5**, 93–105.
- McGuinness, T.B. and Kemp, W.M. (1981). *Schistosoma mansoni*: a complement-dependent receptor on adult male parasites. *Experimental Parasitology* **51**, 236–242.
- McLaren, D.J., Clegg, J.A. and Smithers, S.R. (1975). Acquisition of host antigens by young *Schistosoma mansoni* in mice: correlation with failure to bind antibody *in vitro*. *Parasitology* **70**, 67–75.
- McLaren, D.J., Hockley, D.J., Goldring, O.L. and Hammond, B.J. (1978). A freeze fracture study of the developing tegumental outer membrane of *Schistosoma mansoni*. *Parasitology* **76**, 327–348.
- McLaren, D.J. and Incani, R.N. (1982). *Schistosoma mansoni*: acquired resistance of developing schistosomula to immune attack *in vitro*. *Experimental Parasitology* **53**, 285–298.
- Meyer, F., Meyer, H. and Bueding, E. (1970). Lipid metabolism in the parasitic and free-living flatworms, *Schistosoma mansoni* and *Dugesia dorotocephala*. *Biochimica et Biophysica Acta* **210**, 257–266.
- Morris, G.P. and Threadgold, L.T. (1968). Ultrastructure of the tegument of adult *Schistosoma mansoni*. *Journal of Parasitology* **54**, 15–27.
- Mudad, R. and Telen, M.J. (1996). Biologic functions of blood group antigens. *Current Opinion in Hematology* **3**, 473–479.
- Murrell, K.D., Taylor, D.W., Vannier, W.E. and Dean, D.A. (1978). *Schistosoma mansoni*: analysis of surface membrane carbohydrates using lectins. *Experimental Parasitology* **46**, 247–255.
- Ninno-Smith, R.H. and Standen, O.D. (1963). Phosphomonoesterases of *Schistosoma mansoni*. *Experimental Parasitology* **13**, 305–322.
- Norden, A.P., Aronstein, W.S. and Strand, M. (1982). *Schistosoma mansoni*: identification, characterization, and purification of the spine glycoprotein by monoclonal antibody. *Experimental Parasitology* **54**, 432–442.
- Nyame, A.K., Pilcher, J.B., Tsang, V.C. and Cummings, R.D. (1996). *Schistosoma mansoni* infection in humans and primates induces cytolytic antibodies to surface Le(x) determinants on myeloid cells. *Experimental Parasitology* **82**, 191–200.
- Nyame, A.K., Kwar, Z.S. and Cummings, R.D. (2004). Antigenic glycans in parasitic infections: implications for vaccines and diagnostics. *Archives of Biochemistry and Biophysics* **426**, 182–200.
- Osman, A., Niles, E.G. and LoVerde, P.T. (2001). Identification and characterization of a Smad2 homologue from *Schistosoma mansoni*, a transforming growth factor-beta signal transducer. *Journal of Biological Chemistry* **276**, 10072–10082.
- Ouaissi, M.A., Cornette, J. and Capron, A. (1984). Occurrence of fibronectin antigenic determinants on *Schistosoma mansoni* lung schistosomula and adult worms. *Parasitology* **88**(Pt 1), 85–96.

- Parizade, M., Arnon, R., Lachmann, P.J. and Fishelson, Z. (1994). Functional and antigenic similarities between a 94-kD protein of *Schistosoma mansoni* (SCIP-1) and human CD59. *Journal of Experimental Medicine* **179**, 1625–1636.
- Pax, R.A., Siefker, C. and Bennett, J.L. (1984). *Schistosoma mansoni*: differences in acetylcholine, dopamine, and serotonin control of circular and longitudinal parasite muscles. *Experimental Parasitology* **58**, 314–324.
- Payares, G., Kelly, C., Smithers, S.R. and Evans, W.H. (1985a). Evidence that the 32, 38 and 20 kilodalton surface antigens of schistosomula and schistosomes are a family of conserved proteins. *Molecular and Biochemical Parasitology* **17**, 115–130.
- Payares, G., Smithers, S.R. and Evans, W.H. (1984). Purification and topographical location of tegumental alkaline phosphatase from adult *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **13**, 343–360.
- Payares, G., McLaren, D.J., Evans, W.H. and Smithers, S.R. (1985b). Antigenicity and immunogenicity of the tegumental outer membrane of adult *Schistosoma mansoni*. *Parasite Immunology* **7**, 45–61.
- Payares, G., McLaren, D.J., Evans, W.H. and Smithers, S.R. (1985c). Changes in the surface antigen profile of *Schistosoma mansoni* during maturation from cercaria to adult worm. *Parasitology* **91**(Pt 1), 83–99.
- Pearce, E.J., Basch, P.F. and Sher, A. (1986a). Evidence that the reduced surface antigenicity of developing *Schistosoma mansoni* schistosomula is due to antigen shedding rather than host molecule acquisition. *Parasite Immunology* **8**, 79–94.
- Pearce, E.J., James, S.L., Dalton, J., Barrall, A., Ramos, C., Strand, M. and Sher, A. (1986b). Immunochemical characterization and purification of Sm-97, a *Schistosoma mansoni* antigen monospecifically recognized by antibodies from mice protectively immunized with a nonliving vaccine. *Journal of Immunology* **137**, 3593–3600.
- Pearce, E.J. and Sher, A. (1989). Three major surface antigens of *Schistosoma mansoni* are linked to the membrane by glycosylphosphatidylinositol. *Journal of Immunology* **142**, 979–984.
- Pearce, E.J., Hall, B.F. and Sher, A. (1990). Host-specific evasion of the alternative complement pathway by schistosomes correlates with the presence of a phospholipase C-sensitive surface molecule resembling human decay accelerating factor. *Journal of Immunology* **144**, 2751–2756.
- Pearce, E.J., Magee, A.I., Smithers, S.R. and Simpson, A.J. (1991). Sm25, a major schistosome tegumental glycoprotein, is dependent on palmitic acid for membrane attachment. *Embo Journal* **10**, 2741–2746.
- Perez, H. and Terry, R.J. (1973). The killing of adult *Schistosoma mansoni* *in vitro* in the presence of antisera to host antigenic determinants and peritoneal cells. *International Journal for Parasitology* **3**, 499–503.
- Podesta, R.B., Boyce, J.F., Schurch, S. and McDiarmid, S.S. (1987). Differential adhesive properties of the surface of human blood flukes

- Schistosoma mansoni*. Sexual, regional, and developmental differences in interfacial free energies and structure of glycocalyx. *Cell Biophysics* **10**, 23–31.
- Pujol, F.H. and Cesari, I.M. (1990). Antigenicity of adult *Schistosoma mansoni* alkaline phosphatase. *Parasite Immunology* **12**, 189–198.
- Pujol, F.H., Liprandi, F., Rodriguez, M. and Cesari, I.M. (1990). Production of a mouse monoclonal antibody against the alkaline phosphatase of adult *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **40**, 43–52.
- Racoosin, E.L., Davies, S.J. and Pearce, E.J. (1999). Caveolae-like structures in the surface membrane of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **104**, 285–297.
- Rasmussen, K.R. and Kemp, W.M. (1987). *Schistosoma mansoni*: interactions of adult parasites with the complement system. *Parasite Immunology* **9**, 235–248.
- Redman, C.A. and Kusel, J.R. (1996). Distribution and biophysical properties of fluorescent lipids on the surface of adult *Schistosoma mansoni*. *Parasitology* **113**(Pt 2), 137–143.
- Redman, C.A., Kennington, S., Spathopoulou, T. and Kusel, J.R. (1997). Interconversion of sphingomyelin and ceramide in adult *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **90**, 145–153.
- Remoortere, A., Hokke, C.H., van Dam, G.J., van Die, I., Deelder, A.M. and van den Eijnden, D.H. (2000). Various stages of schistosoma express Lewis(x), LacdiNAc, GalNAcbeta1-4 (Fucalpha1-3)GlcNAc and GalNAcbeta1-4(Fucalpha1-2Fucalpha1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. *Glycobiology* **10**, 601–609.
- Reynolds, S.R., Shoemaker, C.B. and Harn, D.A. (1992). T and B cell epitope mapping of SM23, an integral membrane protein of *Schistosoma mansoni*. *Journal of Immunology* **149**, 3995–4001.
- Ribeiro, F., Coelho, P.M., Vieira, L.Q., Powell, K. and Kusel, J.R. (1998). Membrane internalization processes in different stages of *Schistosoma mansoni* as shown by a styryl dye (Frei Mao 1-43). *Parasitology* **116**, 51–59.
- Richter, D. and Harn, D.A. (1993). Candidate vaccine antigens identified by antibodies from mice vaccinated with 15- or 50-kilorad-irradiated cercariae of *Schistosoma mansoni*. *Infection and Immunity* **61**, 146–154.
- Richter, D., Incani, R.N. and Harn, D.A. (1993a). Isotype responses to candidate vaccine antigens in protective sera obtained from mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. *Infection and Immunity* **61**, 3003–3011.
- Richter, D., Reynolds, S.R. and Harn, D.A. (1993b). Candidate vaccine antigens that stimulate the cellular immune response of mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. *Journal of Immunology* **151**, 256–265.

- Riengrojpitak, S., Vojvodic, M., Boot, C. and Wilson, R.A. (1989). Reactivity of anti-tegument monoclonal antibodies with target epitopes in different worm tissues and developmental stages of *Schistosoma mansoni*. *Parasitology* **98**, 213–225.
- Roberts, S.M., Aitken, R., Vojvodic, M., Wells, E. and Wilson, R.A. (1983). Identification of exposed components on the surface of adult *Schistosoma mansoni* by lactoperoxidase-catalysed iodination. *Molecular and Biochemical Parasitology* **9**, 129–143.
- Robertson, N.P. and Cain, G.D. (1985). Isolation and characterization of glycosaminoglycans from *Schistosoma mansoni*. *Comparative Biochemistry and Physiology B* **82**, 299–306.
- Rogers, M.V. and McLaren, D.J. (1987). Analysis of total and surface membrane lipids of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **22**, 273–288.
- Rogers, M.V., Henkle, K.J., Fidge, N.H. and Mitchell, G.F. (1989). Identification of a multispecific lipoprotein receptor in adult *Schistosoma japonicum* by ligand blotting analyses. *Molecular and Biochemical Parasitology* **35**, 79–88.
- Rogers, M.V., Quilici, D., Mitchell, G.F. and Fidge, N.H. (1990). Purification of a putative lipoprotein receptor from *Schistosoma japonicum* adult worms. *Molecular and Biochemical Parasitology* **41**, 93–100.
- Rumjanek, F.D. and McLaren, D.J. (1981). *Schistosoma mansoni*: modulation of schistosomular lipid composition by serum. *Molecular and Biochemical Parasitology* **3**, 239–252.
- Rumjanek, F.D., Campos, E.G. and Afonso, L.C. (1988). Evidence for the occurrence of LDL receptors in extracts of schistosomula of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **28**, 145–152.
- Ruppel, A. and McLaren, D.J. (1986). *Schistosoma mansoni*: surface membrane stability *in vitro* and *in vivo*. *Experimental Parasitology* **62**, 223–236.
- Ruppel, A., McLaren, D.J., Diesfeld, H.J. and Rother, U. (1984). *Schistosoma mansoni*: escape from complement-mediated parasitocidal mechanisms following percutaneous primary infection. *European Journal of Immunology* **14**, 702–708.
- Sands, W.A. and Kusel, J.R. (1992). Changes in the lateral diffusion of fluorescent lipid analogues in the surface membrane of adult male *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **53**, 233–239.
- Santoro, F., Ouassii, M.A., Pestel, J. and Capron, A. (1980). Interaction between *Schistosoma mansoni* and the complement system: binding of C1q to schistosomula. *Journal of Immunology* **124**, 2886–2891.
- Sauma, S.Y. and Strand, M. (1990). Identification and characterization of glycosylphosphatidylinositol-linked *Schistosoma mansoni* adult worm immunogens. *Molecular and Biochemical Parasitology* **38**, 199–209.
- Sauma, S.Y., Tanaka, T.M. and Strand, M. (1991). Selective release of a glycosylphosphatidylinositol-anchored antigen from the surface

- of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **46**, 73–80.
- Saunders, N., Wilson, R.A. and Coulson, P.S. (1987). The outer bilayer of the adult schistosome tegument surface has a low turnover rate *in vitro* and *in vivo*. *Molecular and Biochemical Parasitology* **25**, 123–131.
- Schechtman, D., Tarrab-Hazdai, R. and Arnon, R. (2001). The 14-3-3 protein as a vaccine candidate against schistosomiasis. *Parasite Immunology* **23**, 213–217.
- Schmidt, J. (1995). Glycans with N-acetylglucosamine type 2-like residues covering adult *Schistosoma mansoni*, and glycomimesis as a putative mechanism of immune evasion. *Parasitology* **111**(Pt 3), 325–336.
- Schmidt, J., Bodor, O., Gohr, L. and Kunz, W. (1996). Paramyosin isoforms of *Schistosoma mansoni* are phosphorylated and localized in a large variety of muscle types. *Parasitology* **112**(Pt 5), 459–467.
- Sell, K.W. and Dean, D.A. (1972). Surface antigens on *Schistosoma mansoni*. I. Demonstration of host antigens on schistosomula and adult worms using the mixed antiglobulin test. *Clinical Experimental Immunology* **12**, 315–324.
- Senft, A.W., Philpott, D.E. and Pelofsky, A.H. (1961). Electron microscopy of the integument, flame cells, and gut of *Schistosoma mansoni*. *Journal of Parasitology* **47**, 217–229.
- Shah, J. and Ramasamy, R. (1982). Surface antigens on cercariae, schistosomula and adult worms of *Schistosoma mansoni*. *International Journal for Parasitology* **12**, 451–461.
- Sher, A., Hall, B.F. and Vadas, M.A. (1978). Acquisition of murine major histocompatibility complex gene products by schistosomula of *Schistosoma mansoni*. *Journal of Experimental Medicine* **148**, 46–57.
- Shi, F., Zhang, Y., Ye, P., Lin, J., Cai, Y., Shen, W., Bickle, Q.D. and Taylor, M.G. (2001). Laboratory and field evaluation of *Schistosoma japonicum* DNA vaccines in sheep and water buffalo in China. *Vaccine* **20**, 462–467.
- Shoemaker, C., Gross, A., Gebremichael, A. and Harn, D. (1992). cDNA cloning and functional expression of the *Schistosoma mansoni* protective antigen triose-phosphate isomerase. *Proceedings of the National Academy of Sciences USA* **89**, 1842–1846.
- Siddiqui, A.A., Podesta, R.B. and Clarke, M.W. (1991). *Schistosoma mansoni*: characterization and identification of calcium-binding proteins associated with the apical plasma membrane and envelope. *Experimental Parasitology* **72**, 63–68.
- Silk, M.H., Spence, I.M. and Gear, J.H. (1969). Ultrastructural studies of the blood fluke *Schistosoma mansoni*. I. The integument. *South African Journal of Medical Science* **34**, 1–10.
- Silva, E.E., Clarke, M.W. and Podesta, R.B. (1993). Characterization of a C3 receptor on the envelope of *Schistosoma mansoni*. *Journal of Immunology* **151**, 7057–7066.

- Simpson, A.J. and Smithers, S.R. (1980). Characterization of the exposed carbohydrates on the surface membrane of adult *Schistosoma mansoni* by analysis of lectin binding. *Parasitology* **81**, 1–15.
- Simpson, A.J., Correa-Oliveira, R., Smithers, S.R. and Sher, A. (1983a). The exposed carbohydrates of schistosomula of *Schistosoma mansoni* and their modification during maturation *in vivo*. *Molecular and Biochemical Parasitology* **8**, 191–205.
- Simpson, A.J., Singer, D., McCutchan, T.F., Sacks, D.L. and Sher, A. (1983b). Evidence that schistosome MHC antigens are not synthesized by the parasite but are acquired from the host as intact glycoproteins. *Journal of Immunology* **131**, 962–965.
- Skelly, P.J. (2004). Intravascular schistosomes and complement. *Trends in Parasitology* **20**, 370–374.
- Skelly, P.J. and Shoemaker, C.B. (1996). Rapid appearance and asymmetric distribution of glucose transporter SGTP4 at the apical surface of intra-mammalian-stage *Schistosoma mansoni*. *Proceedings of the National Academy of Sciences USA* **93**, 3642–3646.
- Skelly, P.J. and Shoemaker, C.B. (2000). Induction cues for tegument formation during the transformation of *Schistosoma mansoni* cercariae. *International Journal for Parasitology* **30**, 625–631.
- Skelly, P.J. and Shoemaker, C.B. (2001). The *Schistosoma mansoni* host-interactive tegument forms from vesicle eruptions of a cyton network. *Parasitology* **122**(Pt 1), 67–73.
- Skelly, P.J., Tielens, A.G.M. and Shoemaker, C.B. (1998). Glucose transport and metabolism in mammalian-stage schistosomes. *Parasitology Today* **14**, 402–406.
- Skelly, P.J., Pfeiffer, R., Verrey, F. and Shoemaker, C.B. (1999). SPRM11c, a heterodimeric amino acid permease light chain of the human parasitic platyhelminth, *Schistosoma mansoni*. *Parasitology* **119**(Pt 6), 569–576.
- Skelly, P.J., Dougan, P.M., Maule, A., Day, T.A. and Shoemaker, C.B. (2001). Cloning and characterization of a muscle isoform of a Na,K-ATPase alpha subunit (SNaK1) from *Schistosoma mansoni*. *Parasitology* **123**, 277–284.
- Smith, H.V. and Kusel, J.R. (1979). The acquisition of antigens in the intercellular substance of mouse skin by schistosomula of *Schistosoma mansoni*. *Clinical Experimental Immunology* **36**, 430–435.
- Smith, J.H., Reynolds, E.S. and Von Lichtenberg, F. (1969). The integument of *Schistosoma mansoni*. *American Journal of Tropical Medicine and Hygiene* **18**, 28–49.
- Smithers, S.R., Terry, R.J. and Hockley, D.J. (1969). Host antigens in schistosomiasis. *Proceedings of the Royal Society of London (B) Biological Sciences* **171**, 483–494.
- Smithers, S.R., Hackett, F., Ali, P.O. and Simpson, A.J. (1989). Protective immunization of mice against *Schistosoma mansoni* with purified adult worm surface membranes. *Parasite Immunology* **11**, 301–318.

- Snary, D., Smith, M.A. and Clegg, J.A. (1980). Surface proteins of *Schistosoma mansoni* and their expression during morphogenesis. *European Journal of Immunology* **10**, 573–575.
- Sobhon, P., Upatham, E.S. and McLaren, D.J. (1984). Topography and ultrastructure of the tegument of adult *Schistosoma mekongi*. *Parasitology* **89**(Pt 3), 511–521.
- Sogandares-Bernal, F. (1976). Immunoglobulins attached to and in the integument of adult *Schistosoma mansoni* Sambon 1907, from first infection of CF mice. *Journal of Parasitology* **62**, 222–226.
- Soisson, L.M., Masterson, C.P., Tom, T.D., McNally, M.T., Lowell, G.H. and Strand, M. (1992). Induction of protective immunity in mice using a 62-kDa recombinant fragment of a *Schistosoma mansoni* surface antigen. *Journal of Immunology* **149**, 3612–3620.
- Sun, W., Liu, S., Brindley, P.J. and McManus, D.P. (1999). Bacterial expression and characterization of functional recombinant triosephosphate isomerase from *Schistosoma japonicum*. *Protein Expression and Purification* **17**, 410–413.
- Suri, P.K., Goldberg, M., Madikizela, M., Petzke, M.M., Bungiro, R.D., Jr., Davies, S.J., Chakraborty, B., Nguyen, K.B., McCray, J.W., Jr. and Knopf, P.M. (1997). Evaluation of recombinant protein r140, a polypeptide segment of tegumental glycoprotein Sm25, as a defined antigen vaccine against *Schistosoma mansoni*. *Parasite Immunology* **19**, 515–529.
- Tanaka, T.M., Skubitz, A.P. and Strand, M. (1993). *Schistosoma*: a 200-kDa chemotherapeutic target antigen is differentially localized in African vs Oriental species. *Experimental Parasitology* **76**, 293–301.
- Tarleton, R.L. and Kemp, W.M. (1981). Demonstration of IgG-Fc and C3 receptors on adult *Schistosoma mansoni*. *Journal of Immunology* **126**, 379–384.
- Tarrab-Hazdai, R., Levi-Schaffer, F., Smolarsky, M. and Arnon, R. (1984). Acetylcholinesterase of *Schistosoma mansoni*: antigenic cross-reactivity with *Electrophorus electricus* and its functional implications. *European Journal of Immunology* **14**, 205–209.
- Tavares, C.A., Cordeiro, M.N., Mota-Santos, T.A. and Gazzinelli, G. (1980). Artificially transformed schistosomula of *Schistosoma mansoni*: mechanism of acquisition of protection against antibody-mediated killing. *Parasitology* **80**, 95–104.
- Tavares, C.A., Soares, R.C., Coelho, P.M. and Gazzinelli, G. (1978). *Schistosoma mansoni*: evidence for a role of serum factors in protecting artificially transformed schistosomula against antibody-mediated killing *in vitro*. *Parasitology* **77**, 225–243.
- Taylor, J.B., Vidal, A., Torpier, G., Meyer, D.J., Roitsch, C., Balloul, J.M., Southan, C., Sondermeyer, P., Pemble, S., Lecocq, J.P., Capron, A. and Ketterer, B. (1988). The glutathione transferase activity and tissue

- distribution of a cloned Mr28 K protective antigen of *Schistosoma mansoni*. *Embo Journal* **7**, 465–472.
- Tempone, A.J., Bianconi, M.L. and Rumjanek, F.D. (1997). The interaction of human LDL with the tegument of adult *Schistosoma mansoni*. *Molecular and Cellular Biochemistry* **177**, 139–144.
- Threadgold, L.T. (1984). Parasitic platyhelminths. In: *Biology of the Integument* (J. Bereiter-Hahn, A.G. Matolsky and K.S. Richards, eds), pp. 132–191. Berlin: Springer.
- Torpier, G. and Capron, A. (1980). Intramembrane particle movements associated with binding of lectins on *Schistosoma mansoni* surface. *Journal of Ultrastructural Research* **72**, 325–335.
- Torpier, G., Capron, A. and Ouaisi, M.A. (1979a). Receptor for IgG(Fc) and human beta2-microglobulin on *S. mansoni* schistosomula. *Nature* **278**, 447–449.
- Torpier, G., Ouaisi, M.A. and Capron, A. (1979b). Freeze-fracture study of immune-induced *Schistosoma mansoni* membrane alterations. I. Complement-dependent damage in the presence of antisera to host antigenic determinants. *Journal of Ultrastructural Research* **67**, 276–287.
- Torres, C.R., Vasconcelos, E.G., Ferreira, S.T. and Verjovski-Almeida, S. (1998). Divalent cation dependence and inhibition of *Schistosoma mansoni* ATP diphosphohydrolase by fluorosulfonylbenzoyl adenosine. *European Journal of Biochemistry* **251**, 516–521.
- Trottein, F., Nutten, S., Papin, J.P., Lepointier, C., Poulain-Godefroy, O., Capron, A. and Capron, M. (1997). Role of adhesion molecules of the selectin-carbohydrate families in antibody-dependent cell-mediated cytotoxicity to schistosome targets. *Journal of Immunology* **159**, 804–811.
- van Balkom, B.W., van Gestel, R.A., Brouwers, J.F., Krijgsveld, J., Tielens, A.G., Heck, A.J. and van Hellemond, J.J. (2005). Mass spectrometric analysis of the *Schistosoma mansoni* tegumental sub-proteome. *Journal of Proteome Research* **4**, 958–966.
- van Remoortere, A., van Dam, G.J., Hokke, C.H., van den Eijnden, D.H., van Die, I. and Deelder, A.M. (2001). Profiles of immunoglobulin M (IgM) and IgG antibodies against defined carbohydrate epitopes in sera of *Schistosoma*-infected individuals determined by surface plasmon resonance. *Infection and Immunity* **69**, 2396–2401.
- Vasconcelos, E.G., Nascimento, P.S., Meirelles, M.N., Verjovski-Almeida, S. and Ferreira, S.T. (1993). Characterization and localization of an ATP-diphosphohydrolase on the external surface of the tegument of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **58**, 205–214.
- Vasconcelos, E.G., Ferreira, S.T., Carvalho, T.M., Souza, W., Kettlun, A.M., Mancilla, M., Valenzuela, M.A. and Verjovski-Almeida, S. (1996). Partial purification and immunohistochemical localization of ATP diphosphohydrolase from *Schistosoma mansoni*. Immunological cross-reactivities

- with potato apyrase and *Toxoplasma gondii* nucleoside triphosphate hydrolase. *Journal of Biological Chemistry* **271**, 22139–22145.
- Verjovski-Almeida, S., DeMarco, R., Martins, E.A., Guimaraes, P.E., Ojopi, E.P., Paquola, A.C., Piazza, J.P., Nishiyama, M.Y., Jr., Kitajima, J.P., Adamson, R.E., Ashton, P.D., Bonaldo, M.F., Coulson, P.S., Dillon, G.P., Farias, L.P., Gregorio, S.P., Ho, P.L., Leite, R.A., Malaquias, L.C., Marques, R.C., Miyasato, P.A., Nascimento, A.L., Ohlweiler, F.P., Reis, E.M., Ribeiro, M.A., Sa, R.G., Stukart, G.C., Soares, M.B., Gargioni, C., Kawano, T., Rodrigues, V., Madeira, A.M., Wilson, R.A., Menck, C.F., Setubal, J.C., Leite, L.C. and Dias-Neto, E. (2003). Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. *Nature Genetics* **35**, 148–157.
- Waine, G.J., Alarcon, J.B., Qiu, C. and McManus, D.P. (1999). Genetic immunization of mice with DNA encoding the 23 kDa transmembrane surface protein of *Schistosoma japonicum* (Sj23) induces antigen-specific immunoglobulin G antibodies. *Parasite Immunology* **21**, 377–381.
- Wheater, P.R. and Wilson, R.A. (1976). The tegument of *Schistosoma mansoni*: a histochemical investigation. *Parasitology* **72**, 99–109.
- William, S., Botros, S., Ismail, M., Farghally, A., Day, T.A. and Bennett, J.L. (2001). PZQ-induced tegumental damage *in vitro* is diminished in schistosomes derived from PZQ-resistant infections. *Parasitology* **122**(Pt 1), 63–66.
- Wilson, R.A. and Barnes, P.E. (1974a). An *in vitro* investigation of dynamic processes occurring in the schistosome tegument, using compounds known to disrupt secretory processes. *Parasitology* **68**, 259–270.
- Wilson, R.A. and Barnes, P.E. (1974b). The tegument of *Schistosoma mansoni*: observations on the formation, structure and composition of cytoplasmic inclusions in relation to tegument function. *Parasitology* **68**, 239–258.
- Wilson, R.A. and Barnes, P.E. (1977). The formation and turnover of the membranocalyx on the tegument of *Schistosoma mansoni*. *Parasitology* **74**, 61–71.
- Wilson, R.A. and Barnes, P.E. (1979). Synthesis of macromolecules by the epithelial surfaces of *Schistosoma mansoni*: an autoradiographic study. *Parasitology* **78**, 295–310.
- Wright, M.D., Henkle, K.J. and Mitchell, G.F. (1990). An immunogenic Mr 23,000 integral membrane protein of *Schistosoma mansoni* worms that closely resembles a human tumor-associated antigen. *Journal of Immunology* **144**, 3195–3200.
- Wright, M.D., Melder, A.M., Davern, K.M. and Mitchell, G.F. (1991). Serologic reactivities of the 23-kDa integral membrane proteins of schistosomes. *Journal of Immunology* **147**, 4338–4342.
- Xu, X. and Caulfield, J.P. (1992). Characterization of human low density lipoprotein binding proteins on the surface of schistosomula of *Schistosoma mansoni*. *European Journal of Cell Biology* **57**, 229–235.

- Zhang, Y., Taylor, M.G. and Bickle, Q.D. (1998). *Schistosoma japonicum* myosin: cloning, expression and vaccination studies with the homologue of the *S. mansoni* myosin fragment IrV-5. *Parasite Immunology* **20**, 583–594.
- Zhong, C., Skelly, P.J., Leafer, D., Cohn, R.G., Caulfield, J.P. and Shoemaker, C.B. (1995). Immunologyocalization of a *Schistosoma mansoni* facilitated diffusion glucose transporter to the basal, but not the apical, membranes of the surface syncytium. *Parasitology* **110**(Pt 4), 383–394.
- Zhou, Y. and Podesta, R.B. (1989). Effects of serotonin (5HT) and complement C3 on the synthesis of the surface membrane precursors of adult *Schistosoma mansoni*. *Journal of Parasitology* **75**, 333–343.

Immunology and Pathology of Intestinal Trematodes in Their Definitive Hosts

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ABSTRACT

This review examines the significant literature on the immunology and pathology of intestinal trematodes in their definitive hosts. We emphasize information on selected species in six families for which the literature on these topics is extensive. The families are Brachylaimidae, Diplostomidae, Echinostomatidae, Gymnophallidae, Heterophyidae, and Paramphistomidae. For most of these families, coverage is considered under the following headings: (i) background; (ii) pathology of the infection; (iii) immunology of the infection; (iv) immunodiagnosis; and (v) human infection. Some of these heading have been subdivided further, based on the literature available on a particular topic. Following this coverage, we include a final section on the important topical literature on selected trematodes in families other than the six mentioned above.

1. INTRODUCTION

In global terms, intestinal trematodes are the commonest parasitic infections in humans and animals. Although less associated with mortality than other groups of parasites, intestinal trematodes are responsible for significant morbidity. In human terms, a large number of species of intestinal trematodes transmitted by food have been reported. Numerous genera of digenetic trematodes, of the families Brachylaimidae,

Diplostomidae, Echinostomatidae, Fasciolidae, Gymnophallidae, Heterophyidae, Lecithodendriidae, Microphallidae, Nanophyetidae, Paramphistomatidae, Plagiorchiidae, and Strigeidae, comprising over 70 species, cause intestinal infection in humans. Table 1 shows a list of the families, genera, and most relevant species implicated in human intestinal trematodiasis (Yu and Mott, 1994; WHO, 1995; Marty and Andersen, 2000; Fried *et al.*, 2004). All of these species are acquired by humans from the ingestion of fish, mollusks, and arthropods, and the endemicity of the parasites is associated with cultural and eating habits (Table 1).

Moreover, the impact of intestinal trematodes in wildlife is significant and these parasites play an important role as causative agents of disease and even death in wildfowl and other animals. Intestinal trematodiasis have been often correlated with death of animals in the wild (Roscoe and Huffman, 1982, 1983). Apart from the interest of intestinal trematodes as causative agents of diseases, these parasites have served as useful models in experimental parasitology for years. They have been used as experimental animal models at all levels of organization, from molecular to organismic.

Most of the current knowledge on the pathology and immunology of intestinal helminths in their definitive hosts is based on studies of nematodes. These studies, together with recent advances in basic immunology, have served to gain further insight in the immune response against intestinal helminths (Finkelman *et al.*, 1997; Maizels and Holland, 1998; Garside *et al.*, 2000; Lawrence, 2003; Maizels and Yazdanbakhsh, 2003; Hayes *et al.*, 2004; Khan and Collins, 2004; Mulcahy *et al.*, 2004). In spite of these studies, the immune mechanisms involved in parasite rejection or, in contrast, the establishment of chronic infections are not understood. In this context, application of recent advances to the study of intestinal trematodiasis may provide important information to the understanding of host-parasite relationships in intestinal helminth infections. The use of these helminths as experimental models may facilitate new studies to determine the elicitation of the responses that facilitate intestinal helminth resistance (Toledo and Fried, 2005). Furthermore, this may be of importance in understanding the role of intestinal trematodes in host populations in the wild.

Table 1 Epidemiological characteristics of the most relevant human intestinal trematodiasis

Genus and most relevant species	Disease	Source of infection	Geographical distribution
Family Brachylaimidae <i>Brachylaima</i> <i>B. cribbi</i>	Brachylaimidiasis	Terrestrial snails	Australia
Family Diplostomidae <i>Neodiplostomum</i> <i>N. seoulense</i>	Diplostomidiasis	Frogs, terrestrial snakes	Korea
Family Echinostomidae <i>Echinochasmus</i> <i>E. perfoliatus</i>	Echinostomiasis	Freshwater fish	Hungary, Italy, Romania, Japan, Taiwan, China
<i>Echinoparyphium</i> <i>E. recurvatum</i>		Tadpoles, frogs, snails	Taiwan, Indonesia, Egypt
<i>Echinostoma</i> <i>E. cinetorchis</i>		Loach, tadpoles, frogs, salamanders	Japan, Taiwan, Korea, Indonesia
<i>E. lindoense</i>		Mussels	Indonesia
<i>E. malayanum</i>		Aquatic snails	Singapore, Malaysia, Thailand, Indonesia, Philippines
<i>E. revolutum</i>		Clams, tadpoles	Taiwan, Thailand, China, Indonesia
<i>Episthmium</i> <i>E. caninum</i>		Freshwater fish	Thailand
<i>Euparyphium</i> <i>E. melis</i>		Tadpoles, loach	Romania, China, North America
<i>Himasthla</i> <i>H. muehlensi</i>		Clams	The Americas
<i>Hypoderaeum</i> <i>H. conoideum</i>		Aquatic snails	Thailand
<i>Paryphostomum</i> <i>P. sufratyfex</i>		Aquatic snails	India

Table 1 (Continued)

Genus and most relevant species	Disease	Source of infection	Geographical distribution
Family Fasciolidae <i>Fasciolopsis</i> <i>F. buski</i>	Fasciolopsiasis	Water chestnut, caltrop, roots of lotus, bamboo, other aquatic vegetation	India, Bangladesh, Pakistan, Myanmar, Korea, China, Taiwan, Vietnam, Cambodia, Thailand, Indonesia, Laos
Family Gymnophallidae <i>Gymnophalloides</i> <i>G. seoi</i>	Gymnophallidiasis	Oysters	Korea
Family Heterophyidae <i>Appophalus</i> <i>A. donicus</i>	Heterophyiasis	Fish	USA
<i>Centrocestus</i> <i>C. formosanus</i>	Metagonimiasis	Fish, frogs	China, Taiwan, Philippines
<i>Cryptocotyle</i> <i>C. lingua</i>		<i>Gobius</i> sp., <i>Labrus</i> sp.	Greenland
<i>Diorchitrema</i> <i>D. pseudocirratum</i>		Mullet	Hawaii, Philippines
<i>Haplorchis</i> <i>H. taichui</i>		Fish	Philippines, Thailand, Laos, Taiwan, Bangladesh
<i>H. yokogawai</i>		Crustacean, shrimp, mullet	Philippines, Indonesia, Thailand, China, Taiwan
<i>Heterophyes</i> <i>H. heterophyes</i>		Freshwater fish, brackish-water, mullet	Tunisia, Egypt, Sudan, Iran, Turkey, Israel, India, Japan, China, Philippines, Taiwan, Indonesia

(Continued)

Table 1 (Continued)

Genus and most relevant species	Disease	Source of infection	Geographical distribution
<i>Heterophyopsis</i> <i>H. continua</i>		Mullet, perch	Japan, Korea, China
<i>Metagonimus</i> <i>M. yokogawai</i>		Freshwater fish	Spain, Balkans, Siberia, Israel, China, Japan, Korea, Indonesia
<i>Phagicola</i> <i>Phagicola</i> sp.		Mullet	Brazil, USA
<i>Procercovum</i> <i>P. calderoni</i>		Mullet	Africa, China, Philippines
<i>Pygidiopsis</i> <i>P. summa</i>		Mullet, goby	Japan, Korea
<i>Stellantchasmus</i> <i>S. falcatus</i>		Gray mullet	Japan, Philippines, Hawaii, Thailand, Korea
<i>Stichtodora</i> <i>S. fuscata</i>		Mullet, goby	Japan, Korea
Family Lecithodendriidae	Lecithodendriidiasis		
<i>Prosthodendrium</i> <i>P. molenkampii</i>		Insects	Indonesia, Thailand
<i>Phaneropsolus</i> <i>P. bonnei</i>		Insects	Indonesia, Thailand
Family Microphallidae	Microphallidiasis		
<i>Spelotrema</i> <i>S. brevicæca</i>		Crabs, shrimp	Philippines
Family Nanophyetidae	Nanophyetiasis		
<i>Nanophyetus</i> <i>N. salmincola</i>		Salmon, trout	Siberia, USA
Family Paramphistomatidae	Paramphistomatidiasis		
<i>Gastrodiscoides</i>* <i>G. hominis</i>	Gastrodiscoidiasis	Aquatic plants, crayfish, frogs, tadpoles	India, Vietnam, Philippines, Myanmar, Thailand, China, Kazakhstan

Table 1 (Continued)

Genus and most relevant species	Disease	Source of infection	Geographical distribution
<i>Fischoederius</i> <i>F. elongatus</i>	Plagiorchiidiasis	Aquatic plants	China
<i>Watsonius</i> <i>W. watsoni</i>		Vegetation?	Africa
Family Plagiorchiidae <i>Plagiorchis</i> <i>P. muris</i>		Insects, aquatic snails	Japan
Family Strigeidae <i>Cotylurus</i> <i>C. japonicus</i>		Unknown	China

*In the most recent literature, included within the family Gastrodiscidae.

The purpose of this review is to examine the significant literature on pathological and immunological effects of intestinal trematodes in their definitive hosts. Until now, there was no review that approached these subjects comprehensively and information on the topics has been scattered worldwide in numerous scientific journals. The main goal of our review is to acquaint and update readers on the published information on these topics. For this purpose, we have analyzed separately those families of intestinal trematodes for which a sufficient body of data has been published to justify such individual sections. Moreover, we review in a final section some of the scarce information published on species of other families not covered in the earlier sections of the review.

2. BIOLOGY OF THE INTESTINAL TREMATODES

The class Trematoda comprises an important group of parasitic flatworms of medical and veterinary importance and contains numerous species that are the causative agents of human and animal diseases. The systematics of the Trematoda are being investigated and debated thoroughly; the digenetic trematodes, or digeneans, constitute the largest group of platyhelminths. Within their definitive hosts, these

the eggs are fully embryonated, they either hatch in water or are ingested by their respective first intermediate hosts. The eggs that hatch in water release a ciliated larva, the miracidium, which swims searching for the first intermediate host, and penetrates the soft tissues of this host. In other instances, the eggs are ingested by the first intermediate host and miracidia hatch out and penetrate, and localize in an appropriate site.

The first intermediate host of almost all digeneans is a mollusc, and is typically a snail. Within this host, miracidia develop into sporocysts, but occasionally miracidia may give rise directly to rediae. Development of parthenitae (sporocysts and rediae) follows different patterns depending on the digenean species. In some species, the primary sporocyst gives rise to secondary sporocysts which in turn give rise to cercariae. In other instances, primary sporocysts may give rise to either rediae (first generation) that produce cercariae or rediae of a second generation that produces cercariae. The free-swimming cercariae escape from their molluscan host and come in contact with a compatible second intermediate host, often invertebrate or even a vertebrate. Cercariae actively penetrate the host's body and encyst as metacercariae: the metacercaria is the infective stage for the definitive host. Numerous invertebrates and poikilothermal vertebrates (amphibians, annelids, arthropods, fish, molluscs) serve as second intermediate hosts of intestinal digenetic species but some of these also use their snail first intermediate hosts as their second intermediate hosts. However, in almost all such cases, cercariae first leave the snail and may reenter the same or another snail for encystment. In other life cycles of some intestinal species, the post-cercarial development requires cercarial encystation on vegetation.

The definitive host is a vertebrate, which becomes infected when metacercariae are ingested, either with the intermediate host or on vegetation. Within the definitive host, the encysted metacercaria excysts in the intestine and gradually matures into an adult.

The life cycle of some strigeoids (e.g., genus *Alaria* that includes several species, all of which are parasitic in the small intestine of carnivorous mammals) involves an additional unencysted stage intermediate between the cercaria and metacercaria stage and referred to as the mesocercaria. The cercariae penetrate tadpoles or adult frogs

and develop into mesocercariae, and if these hosts are eaten by their definitive hosts, the parasite develops into a diplostomulum metacercaria, and finally into an intestinal adult. Moreover, in this life cycle, paratenic hosts (e.g., the water snake), harboring mesocercariae are also involved, and transmammary infection of paratenic and definitive hosts with *Alaria* spp. mesocercariae have been reported (Shoop and Corkum, 1984).

3. THE FAMILY BRACHYLAIMIDAE

3.1. Background

The family Brachylaimidae contains numerous species of terrestrial trematodes that infect mammals, birds, and reptiles (Gibson and Bray, 1994). *Brachylaima* is the most representative genus within this family. This genus has had many synonyms with no less than four spellings having been used in the literature (Yamaguti, 1971; Kamiya and Machida, 1977). Many of the species have been poorly described, with incomplete life cycles and insufficient detailed information for accurate identification. This problem is compounded by the morphological similarity of many of the adult worms (Figure 2). Species of *Brachylaima* follow a three-host terrestrial life cycle (Yamaguti, 1975). The first and second intermediate hosts of brachylaimids are either the same or two different species of terrestrial snail species. The definitive host can be either a mammal or a bird. Humans have also been reported as an incidental definitive host for one species of the genus *Brachylaima*, i.e., *B. cribbi* (Butcher *et al.*, 1996, 1998).

3.2. Immunology of the Infections

The complexity of the life cycles of *Brachylaima* spp. may explain the scarcity of the data on host-parasite relationships in infections with brachylaimids. However, the use of different strains of mice as experimental hosts has allowed for studies on several aspects of the development of these flukes and the immunology of the infections. These studies have been focused on *B. cribbi*.

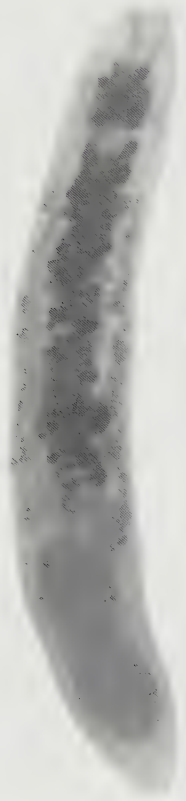


Figure 2 An adult worm of *Brachylaima* sp. from a naturally infected mouse, *Mus musculus*; the worm was stained in Grenacher's Borax Carmine. Scale bar: 0.5 mm.

Butcher *et al.* (2002a) showed that CB57BL 6J is the most susceptible strain of *Mus musculus* to *B. cribbi* infection, having the highest levels of egg release and the longest duration of infection. However, there was variability in susceptibility in relation to gender and maturity of mice (Butcher *et al.*, 2002b). Mature female CB57BL 6J mice were significantly more resistant to *B. cribbi* infection than older mature females and adolescent females with reduced worm burden, fecundity, and egg fertility. In comparison with young male mice, all three parameters were again reduced but this was only statistically significant for egg fertility (Butcher *et al.*, 2002b). These differences in susceptibility were attributed to physiological factors. Butcher *et al.*

(2002b) suggested that sex hormones provided a significant level of protection to *B. cribbi* infection as shown for other intestinal helminths by Zuk and McKean (1996).

B. cribbi evokes significant antibody responses as determined by indirect ELISA using crude adult worm extracts as antigen (Butcher *et al.*, 2003). Anti-*B. cribbi* serum antibody absorbance ratios increased six- to seven-fold by 4 weeks after a primary infection, beyond which a constant level was maintained. Butcher *et al.* (2003) showed that humoral and/or cellular-mediated immunity are important in mediating resistance and influencing the fertility of adult worms. The course of the infection in immunocompetent CB57BL/6J and immunodeficient NOD SCID mice reinfected with *B. cribbi* was assessed by comparing fecal egg release of the reinfected mice with age- and sex-matched mice receiving only a primary infection. In the case of CB57BL/6J mice there were significant differences in the mean fecal eggs per gram of feces and worm fecundity, having lower egg counts and reduced fecundity with the challenge infections. In contrast, no significant differences were observed in NOD SCID mice between primary and challenge infections.

There are no studies on the pathology of infections with brachylaimid species either in natural or experimental infections.

3.3. Immunodiagnosis

An indirect ELISA using whole-worm antigens of *B. cribbi* was developed by Butcher *et al.* (2003). This method was useful to demonstrate that *B. cribbi* induces antibody responses in mice. However, this assay was only used in mice that had no other helminth infections. It, therefore, constitutes a measure of sensitivity rather than specificity. Assessment of cross-reactivity with other helminths would be required before this technique could be considered for sero-diagnosis.

3.4. Human Infections

Of all the brachylaimids studied, only *B. cribbi* has been reported to parasitize humans. These infections were reported from South

Australia (Butcher *et al.*, 1996, 1998; Butcher and Grove, 2001) where the life cycle is maintained between mice, *M. musculus*, and helioid and hygromiid snails (Butcher *et al.*, 1996). Humans often accidentally ingest these snails with vegetables from house gardens or local markets (Butcher *et al.*, 1996, 1998). Infections in humans usually become chronic and can persist as long as 18 months (Butcher *et al.*, 1996). Clinical symptoms depend on the parasite load and heavy infections are associated with diarrhea, abdominal pain, low-grade fever, and fatigue (Butcher *et al.*, 1996; Fried *et al.*, 2004). The immunology of the infection in humans has not been studied.

4. THE FAMILY DIPLOSTOMIDAE

4.1. Background

The family Diplostomidae contains digeneans from numerous orders of birds and mammals. Although there have been some problems in regard to the subdivision of Diplostomidae into subfamilies (Dubois, 1970; Yamaguti, 1971; Gibson, 1996), Niewiadomska (2002) recognized a total of four subfamilies according to host specificity, morphological features and type of metacercariae. These are the Diplostominae, Crassiohialinae, Alariinae, and Codonocephalinae.

In general, species of the Diplostomidae have a three-host life cycle, though some variations of this pattern can be found. Fork-tailed cercariae are produced in sporocysts in the gastropod first intermediate host. The cercariae emerge from the snails and penetrate and form metacercariae in fishes, amphibians, molluscs, and annelids (Hong *et al.*, 1982). In some Diplostomidae, the life cycle is expanded to incorporate four hosts by inclusion of a mesocercaria stage (a form between the cercaria and metacercaria) (see Section 2). Definitive hosts become infected by the ingestion of the second intermediate host or the paratenic host harboring metacercariae. Eggs typically hatch and penetrate the first intermediate host (Cribb *et al.*, 2003).

Experimental studies on the immunobiology and pathology of members of Diplostomidae have only been done on one species, *Neodiplostomum seoulense*, belonging to the Diplostominae. This

species is frequently reported in humans in Korea (Chai and Lee, 1991, 2002) and such reports have stimulated work on this topic. *N. seoulense*, a minute intestinal fluke (Figure 3), was originally described from the small intestine of house rats in the Republic of Korea, and named *Fibricola seoulensis* (Seo *et al.*, 1964). After a debate on its taxonomy, it was renamed *N. seoulense* (Hong and



Figure 3 *Neodiplostomum seoulense*: An adult worm from an experimentally infected rat necropsied at 7 DPI; the worm was stained in acetocarmine. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 100 μ m.

Shoop, 1995). This digenean is an intestinal fluke of humans and animals in Korea (Chai and Lee, 2002). The freshwater snails *Hip-peutis* (*Helicorbis*) *cantori* and *Segmentina* (*polipyxis*) *hemisphaerula* are the first intermediate hosts (Seo *et al.*, 1988; Chung *et al.*, 1996). The second intermediate hosts are tadpoles and frogs, and metacercariae can also be found in snakes which act as paratenic hosts (Hong *et al.*, 1982; Seo *et al.*, 1988). The site of infection in the definitive host is the duodenum but parasites may extend to the jejunum and ileum in heavy infections (Hong *et al.*, 1983). Albino rats, mice, guinea pigs, rabbits, cats, dogs, and chickens are susceptible to experimental *N. seoulense* infection (Hong, 1982; Hong *et al.*, 1983), though albino rats and mice are the most susceptible experimental hosts (Hong *et al.*, 1983), and have been used in experimental studies. In the present section, we review the most relevant data on the immunology and pathology in *N. seoulense* infections.

4.2. Pathology of the Infection

Compared to other intestinal flukes, which only cause mild intestinal problems unless severely infected, *N. seoulense* seems to be more harmful to their definitive hosts, producing severe anatomical and functional damage to the host intestine. For example, *N. seoulense* is highly pathogenic and lethal to laboratory mice (Kook *et al.*, 1998; Chai *et al.*, 2000a).

Lee *et al.* (1985) examined the duodenal histopathology in mice and rats experimentally infected with 500 metacercariae per host of *N. seoulense*. The mucosal findings were similar in both hosts. The worms entrapped the host villi using their concave ventral curvature of the anterior forebody, with the tribocytic organ piercing the villous stroma (Figure 4). The villous changes appeared during the first week of infection and were characterized by shortening, widening, and fusion of the villi. There was also a reduction of the goblet cell numbers adjacent to the worms, hemorrhage, capillary congestion, lymphatic dilation, and inflammatory cell infiltration. The inflammatory cells involved were lymphocytes, plasma cells, eosinophils, and occasional giant cells. No mortality was observed in the infected

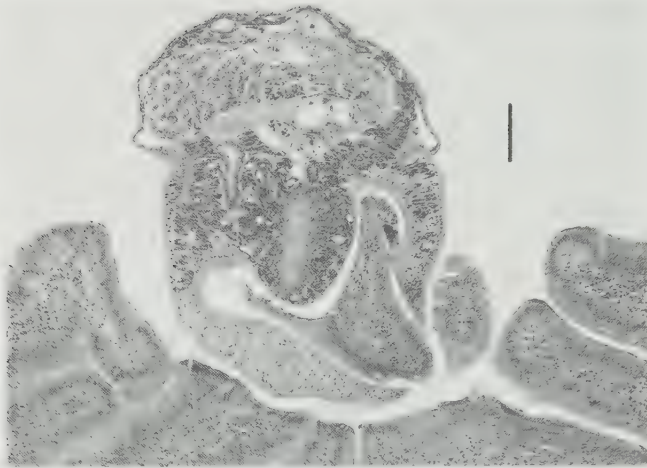


Figure 4 Small intestine of a rat experimentally infected with *Neodiplostomum seoulense* and necropsied at 14 DPI; the preparation was stained with hematoxylin and eosin. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 100 μ m.

mice and rats during the first 2 and 4 weeks post-infection (WPI), respectively. Transmural inflammation was also observed in some of the rats studied. This effect was attributed to ectopic infections as described for the related species *Pharyngostomum cordatum*. Shin *et al.* (2001) showed that metacercariae of *P. cordatum* may pass through the intestinal wall to the peritoneal cavity of rodents, migrating to the diaphragm, intercostal muscles, and other organs such as the lung, heart, and brain. The migration of the metacercariae resulted in considerable damage to the intestinal wall.

Other studies have shown that pathology and lethality of *N. seoulense* infections depend on different factors such as the cyst inoculum and the genetic background of the host. Huh *et al.* (1988) studied the effect of heavy infections in ICR mice. Infections with 1000 metacercariae per host produced a severe course of infection with diarrhea and a gradual weight loss and, finally, a fatal outcome. Death of the infected mice began at 11 days post-infection (DPI), and at 16 DPI all the mice were dead. In contrast, infection with 500 metacercariae per host did not affect mouse survival. The histopathological changes in heavy infections were similar to those described previously by Lee *et al.*

(1985) and were not confined to duodenum but extended to the jejunum and ileum in relation to the higher infective doses. Gross bleeding was also observed in heavily infected mice. Occult blood was observed after 10 DPI; moreover, hemoglobin and mean corpuscular hemoglobin decreased after 12 DPI. Huh *et al.* (1988) suggested that intestinal malabsorption induced by mucosal changes and intestinal bleeding were the main causes of the earlier death in the heavily infected mice. Kook *et al.* (1998) showed that survival of C3H/HeJ mice was inversely related to the dose of metacercariae. The survival of mice each given 25, 50, 100, and 200 metacercariae was 60, 20, 40, and 0%, respectively, on 28 DPI. Kook *et al.* (1998) also found that C57BL 6 mice each infected with 200 metacercariae of *N. seoulense* showed distended intestinal loops and segmental contractions. The length of the intestine was significantly shortened and charcoal meal transit was significantly quicker after 14 DPI compared with controls, suggesting intestinal paralysis.

There are some discrepancies in relation to the reversibility of the intestinal damage produced by *N. seoulense* infections depending on the species and even the strain of the host. Lee *et al.* (1989) examined the mucosal changes after praziquantel treatment of rats, each experimentally infected with 1000 metacercariae. Until day 3 after treatment, the mucosa was severely atrophied, whereas on day 5 post-treatment, long and slender villi sometimes appeared among the fused stout ones. After day 15 post-treatment, the villi were returning to their normal appearance. In contrast, the damage caused by the parasite in the first DPI appeared to be lethal for mice. Kook *et al.* (1998) showed that C57BL 6 mice each infected with 200 metacercariae exhibited diarrhea and 60% died between 3 and 11 days post-treatment with praziquantel; they showed that intestinal contraction and paralysis induced by the parasite were not reversible.

Little is known about the pathogenic mechanisms of *N. seoulense*. However, it is thought that *N. seoulense* may cause mechanical and chemical damage. The attachment of adult worms is very tenacious (Lee *et al.*, 1985). Each worm embraces a villus with its forebody, which is inserted into the intervillous spaces. This may cause injury in the intestinal mucosa. Moreover, the tribocytic organ appears to be an important structure responsible for the mucosal damage; this

organ, which pierces the host villi, secretes alkaline phosphatase, which can lyse the host villi (Huh *et al.*, 1990; Huh and Song, 1993).

4.3. Immunology of the Infection

4.3.1. Manifestations of Resistance to Infection

Immunity against intestinal helminths can be manifested in several ways (Balic *et al.*, 2000). In *N. seoulense* infections in rats, acquired immunity is manifested by earlier local responses and decreased parasite establishment. Yu *et al.* (1995) compared the worm recovery and the mucosal changes between primary infected rats with 500 metacercariae and reinfected rats after treatment with praziquantel. The worm recovery rates were significantly lower in the reinfected group than in the primary infected rats. Furthermore, the villous changes appeared earlier in the challenge infections. In the primary infections, villous atrophy appeared during the first and second (WPI), whereas in the reinfections, the mucosal changes were evident from day 3 post-infection. The earlier occurrence of the intestinal pathology was attributed to an earlier increase and wider distribution of goblet cells in the small intestine in relation to the stimuli produced by the primary infection. Yu *et al.* (1995) also noted differences in alkaline phosphatase and sucrase intestinal activities between the primary and secondary infections. The enzymatic activities were decreased in primary infections and were unchanged in secondary infections. This could be related to the different kinetics of damage and regeneration processes of the intestinal mucosa in each group of rats.

A genetic influence on the resistance to *N. seoulense* infection has been demonstrated. Kook *et al.* (1998) infected two strains of mice (BALB/cA and C3H/HeJ) and their F₁ hybrid with 200 metacercariae per mouse of *N. seoulense*. Although, BALB/cA mice had the greatest worm intensities, this strain was resistant to pathogenesis and mortality due to the infection. All C3H/HeJ and F₁ mice showed marked intestinal paralysis, which were not observed in BALB/cA mice. Moreover, the survival of BALB/cA mice at 29 DPI (87%) was significantly greater than that observed in C3H/HeJ and F₁ mice (0 and

27%, respectively). Chai *et al.* (2000a) suggested that resistance to *N. seoulense* infection was dependent upon H-2 gene product differences. Chai *et al.* (2000a) observed that a greater proportion of C3H/He (H-2k) succumbed to *N. seoulense* infection despite having fewer worms and shorter duration of the infection than BALB/c (H-2a) and C57BL/6 (H-2b) mice.

4.3.2. Effector Mechanisms of the Immune Response

Some studies are on the immune effector mechanisms in mice and rats experimentally infected with *N. seoulense*. However, studies on the relationships between the cellular and humoral responses and the course of the *N. seoulense* infections have not been done. The published studies have been focused on the mucosal mast cells, goblet cells, and the antibody response during infection.

Mucosal mast cells are important effector cells against intestinal helminths (Nawa *et al.*, 1994). Mucosal mastocytosis is T-cell dependent and requires stimulation by Th2 cytokines (IL-3, IL-4, and IL-10) (Abe *et al.*, 1993; Lantz *et al.*, 1998). In the case of *N. seoulense* infections, worm expulsion concomitantly with intestinal mastocytosis (mainly in the duodenum) has been observed in Sprague-Dawley rats (Kho *et al.*, 1990; Shin *et al.*, 2003) and mice (Chai *et al.*, 1998). However, the kinetics and extent of mastocytosis were different in each host species, suggesting that the role of mucosal mast cells is different in mice and rats.

Chai *et al.* (1998) studied the mucosal mast cell responses in BALB/c and C3H mice each infected with 200 metacercariae. The kinetics of mucosal mast cell responses did not show a correlation with worm expulsion and the degree of proliferation had no relationship with the susceptibility of each strain of mice. The highly susceptible strain of BALB c mice showed a greater mucosal mast cell reaction than C3H mice at 7 DPI but they did not expel the worms until 28 DPI. Therefore, proliferation of mucosal mast cell in the two strains of mice was regarded as local host responses rather than a responsible effector mechanism for worm expulsion. Kook *et al.* (1998) confirmed the above results by infecting mast-cell-deficient W^hW^h mice and their

normal littermate $+/+$ mice with 200 metacercariae per mouse of *N. seoulense*. The parasite was highly lethal for both strains of mice, but $+/+$ mice showed a significantly greater level of worm recovery than W/W^v mice. This indicates that mucosal mast cells are not actively involved in the protective mechanisms against *N. seoulense* infections in mice.

In Sprague-Dawley rats, the number of mucosal mast cells peaked at 3 WPI, coinciding with the decline of worm recovery rates (Kho *et al.*, 1990). Thereafter, the mucosal mast cell numbers gradually declined up to 7 WPI. Consequently, Kho *et al.* (1990) suggested a relationship between mastocytosis and worm expulsion. This contrasts with a later study by Shin *et al.* (2003), who examined the effect of antiallergic drugs on intestinal mastocytosis and the expulsion of *N. seoulense* in Sprague-Dawley rats after oral infection with 500 metacercariae per rat. The drugs used were hydroxyzine (a histamine receptor H1 blocker), cimetidine (a H2 blocker), cyclosporin-A (a Th-cell suppressant), and prednisolone (a T- and B-cell suppressant). In the cyclosporin-A- and prednisolone-treated groups, mastocytosis was suppressed but worm expulsion was not delayed with respect to the non-treated infected controls, indicating that mucosal mast cells were not essential for the rejection of the infection. Histamine appears to be more important, since in hydroxyzine- and cimetidine-treated groups, worm expulsion was delayed despite the fact that mastocytosis was similar to that observed in the non-treated infected controls. This is surprising since mucosal mast cells are the main source of histamine (MacGlashan, 2003). These results suggest that intestinal smooth muscle contraction plays a major role in *N. seoulense* expulsion. Hydroxyzine and cimetidine inhibit the smooth muscle contraction by blocking the histamine receptors and, consequently, could delay the worm expulsion regardless of the level of mastocytosis.

The above results suggest the existence of other effector mechanisms in the expulsion of *N. seoulense* from its definitive host. Intestinal goblet cell hyperplasia is a candidate effector mechanism as reported in nematodes (see, e.g., Nawa *et al.*, 1994; Loukas and Prociv, 2001). Goblet cell hyperplasia is thought to be T-cell-dependent, though its regulation is different than that of mucosal mast cell hyperplasia (Balic *et al.*, 2000). Goblet cell hyperplasia plays an important role in the

expulsion of other intestinal trematode infections (see Section 5.3). The intestinal goblet cell hyperplasia in *N. seoulense* infections has been studied in BALB c and C3H mice by Chai *et al.* (1998). Goblet cell hyperplasia was observed in the duodenum of BALB/c mice during the course of the infection but was not seen in C3H mice. Mucin activation was noted in both strains of mice, though more evident in BALB c mice, in spite of the worm recovery rate kinetics in each strain of mice.

As noted with the mucosal mast cell and goblet cell responses, humoral responses do not appear to play a major role in *N. seoulense* infections. *N. seoulense* infection in rats induces the production of systemic specific IgG with a peak at 10 DPI (Kho *et al.*, 1990). The kinetics of IgA in serum and duodenal mucosa and submucosa of BALB c mice was studied by Huh *et al.* (1995). A continuous increase of serum IgA occurred from day 3 post-infection until the end of the experiment at day 28 post-infection. Increased levels of IgA were also detected in the lamina propria and submucosa but these responses were non-specific. These antibody responses were not related to worm expulsion since significant decreases in worm recoveries were not observed during the experiment.

4.3.3. Antigenic Characterization

There are several studies concerning the antigenic composition of *N. seoulense*. These studies have been done mainly to search for the factors determining the pathogenesis of this parasite species.

Lee *et al.* (1997a) examined the localization of *N. seoulense* antigens by immuno-electronmicroscopy using sera from mice immunized with adult crude worm extracts and sera from infected mice. As expected, the results were different with each antibody. Thin sections of *N. seoulense* adult worms treated with the immunized serum showed high concentration of antibody over the rough endoplasmic reticulum of the cells of the tribocytic organ, spermatozoa in the seminal vesicle, microvilli of the cecum, and vitelline follicles. Using the infected serum, the most reactive structure was the vitelline follicle. Lee *et al.* (1997a) suggested that the most antigenic components of the

excretory/secretory products originated from the vitelline follicles as previously described for other intestinal trematodes (Ahn *et al.*, 1991).

Choi *et al.* (1999) showed that the excretory/secretory products and the adult crude worm extracts of *N. seoulense* have proteolytic activity. Furthermore, the same authors purified a 54-kDa protease from crude extracts. The enzyme degraded extracellular matrix proteins such as type I collagen and fibronectin with different cleaving activities. According to the activity observed *in vitro*, this protein could play an important role in parasite survival and pathogenesis *in vivo*. Choi *et al.* (1999) suggested a possible role in nutrient uptake by degrading host tissue proteins.

4.4. Human Infections

At least members of three genera of Diplostomidae (*Alaria*, *Neodiplostomum*, and *Fibricola*) are known to parasitize man (see Table 1). In the case of *Alaria* spp., humans serve as paratenic hosts harboring metacercariae in different tissues (Fernandes *et al.*, 1976; Freeman *et al.*, 1976; Beaver *et al.*, 1977; McDonald *et al.*, 1994; Kramer *et al.*, 1996). At the intestinal level, only *N. seoulense* and *F. cratera* parasitize humans.

Human infections with *N. seoulense* have been reviewed recently (Chai and Lee, 2002; Fried *et al.*, 2004). A total of 28 human cases have been reported in the Republic of Korea, but none in other countries (Chai and Lee, 2002). This species was first implicated when an infected human was found suffering severe enteritis with abdominal pain, fever, diarrhea, fullness, and anorexia (Seo *et al.*, 1982). The patient had a history of eating raw snakes, which appears to be the most important food source for human infections (Hong *et al.*, 1984a, b). Chai and Lee (2002) estimated the total number of human cases as 1000 in the Republic of Korea. There are no available studies on the pathology and immunology of *N. seoulense* infections in humans.

More anecdotal is the human infection with *F. cratera*, a trematode species indigenous to North America. Shoop (1989) reported an

experimental inoculation of a human volunteer producing a patent infection that lasted 40 months. Symptoms exhibited by the volunteer were similar to those described in *N. seoulense* infections.

5. THE FAMILY ECHINOSTOMATIDAE

5.1. Background

The family Echinostomatidae contains a rather heterogeneous group of cosmopolitan and hermaphroditic digeneans that parasitize, as adults, numerous vertebrate hosts of all classes (Kostadinova and Gibson, 2000; Kostadinova, 2005). Adult echinostomatids are predominantly found in birds, but also parasitize mammals and occasionally reptiles and fishes (Figure 5). Their typical site of location is the intestine though species parasitizing other sites also exist. The main distinguishing feature of the Echinostomatidae is the presence of a circumoral collar armed with one or two ventrally interrupted crowns of spines. The pattern of the collar spines is essential for taxonomic purposes (Figure 6).

Members of the Echinostomatidae follow a three-host life cycle. The first intermediate hosts are aquatic snails in which a sporocyst, two generations of rediae, and cercariae develop. Emerged cercariae infect the second intermediate host, which may be several species of snails, clams, frogs, and even fishes. The definitive host becomes infected after ingestion of the second intermediate host harboring the encysted metacercariae (Huffman and Fried, 1990; Fried and Huffman, 1996; Fried and Graczyk, 2004).

The number of genera included within the Echinostomatidae varies considerably (Kostadinova and Gibson, 2000; Kostadinova, 2005). From those genera, species of *Echinostoma* have been the most widely used in studies on pathological and immunological aspects of infection. Comparative studies on the development of a single species of *Echinostoma* in different host species in which the course of the infection differs (see Table 2) have allowed for the analysis of some of the factors that regulate the immunopathology and the immune response in echinostome infections (Toledo and Fried, 2005).

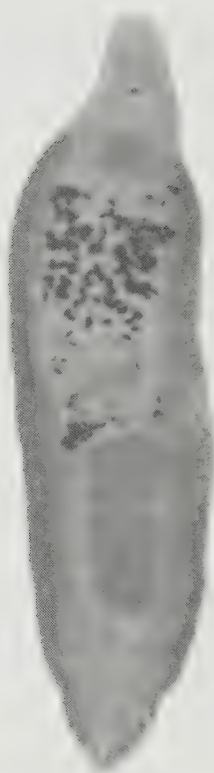


Figure 5 Adult worm of *Echinostoma caproni* from an experimentally infected hamster necropsied at 28 DPI; the worm was stained in Grenacher's Borax Carmine. Scale bar: 1 mm.

The present section is concerned mainly with *Echinostoma trivolvis* and *E. caproni* for which the most literature is available. In several papers, different names have been used for these species due to the taxonomic confusion within the genus *Echinostoma* (see revisions by Kostadinova *et al.*, 2000; Toledo *et al.*, 2000). We use the name currently most accepted for each species, regardless of what name was used in the original paper.

5.2. Pathology of the Infection

The pathology of echinostome infections represents a complex and diverse set of reactions. The complexity is dependent on a wide variety



Figure 6 Low-magnification scanning electron micrographs of the forebody of an adult worm of *Euparyphium albuferensis* showing the spined collar. Scale bar: 50 μ m.

of factors including characteristics of the echinostome species and the nature of the host species. Clinical disease and pathophysiological responses are modulated by the intensity of the infection (Huffman and Fried, 1990). The main signs in heavy infections are weakness, watery diarrhea, weight loss, and unthriftiness.

The pathology of echinostome infections in the wild has been poorly studied. Griffiths *et al.* (1976) reported that geese infected with *Echinostoma revolutum* and *Notocotylus attenuatus* presented lesions in the small intestine consisting of mild hyperemia together with a severe catarrhal enteritis. Kishore and Sinha (1982) reported lymphocytic and monocytic infiltration in the lamina propria of *E. revolutum*-infected ducks. Bandyopadhyay *et al.* (1995) reported that pigs naturally infected with *Artyfechinostomum oraoni* developed

Table 2 Main features of the infection with several species of *Echinostoma* in different rodent models

Species	Host species	Goblet cell hyperplasia	Mast cell hyperplasia	Eosinophilic infiltration	Systemic humoral responses (IgG)	Life span (WPI)
<i>Echinostoma caproni</i>	Mice	—	—	+	Strong	At least 29
	Hamster	ns	ns	+	Strong, slow*	At least 20
	Rat	ns	ns	ns**	Weak	6–8
<i>Echinostoma trivolvis</i>	Mice	+	+	+	Strong	2–4
	Hamster	—	—	—	Weak	At least 15
<i>Echinostoma friedl</i>	Hamster	ns	ns	ns	Strong, slow***	At least 12
	Rat	ns	ns	ns	ns	3–4

ns, not studied.

*Although significant levels of IgG are detected, they appear later than in mice (Simonsen *et al.*, 1991; Toledo *et al.*, 2004b).

**Peripheral eosinophilia has been reported by Hansen *et al.* (1990).

***Although significant levels of IgG are detected, they appear later than in rats (Toledo *et al.*, 2003a, 2004c).

a fatal diarrhea. The necropsy revealed a massive infection with the echinostomatid on a hemorrhagic and edematous mucosa of the jejunum and duodenum extending to the pyloric end of the stomach.

The experimental use of rodent domestic chick models has provided basic knowledge of the pathology of echinostome infections. Kim and Fried (1989) showed that the ileum of domestic chicks experimentally infected with *E. caproni* was dilated and hemorrhagic. Histological lesions were evident in the dilated region along with hypertrophy of the circular musculature. A marked crypt hyperplasia and elongation, and blunting and fusing of the villi were also observed. The non-dilated regions of the ileum appeared normal. Huffman (2000) compared the pathology of *E. revolutum*, *E. trivolvis*, and *E. caproni* in the domestic chick. Damage to the intestinal villi was observed at the site of the worm attachment along with proliferation of goblet cells.

Most of the reports on the intestinal pathology of echinostome infections come from rodent models. *E. caproni* and *E. trivolvis* produce similar intestinal lesions in golden hamsters. Damage has been observed at attachment sites of the echinostomes (Figures 7 and 8).



Figure 7 *Echinostoma caproni*: Small intestine stained with Giemsa of a hamster experimentally infected and necropsied at 14 DPI showing villi destruction and erosion. Scale bar: 0.5 mm.

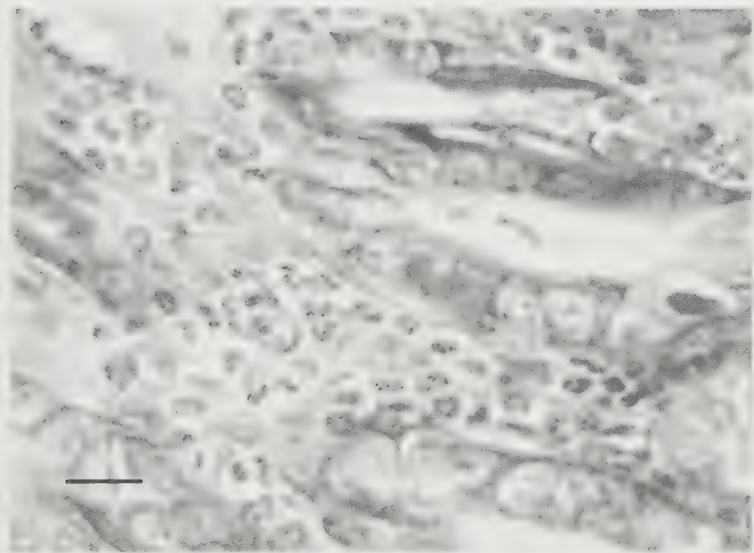


Figure 8 Small intestine stained with alcian blue of a hamster experimentally infected with *Echinostoma caproni* and necropsied at 14 DPI showing a high number of neutrophils. Scale bar: 50 µm.

Such areas showed marked dilation, erosion of the villi, and lymphocytic infiltration (Huffman *et al.*, 1988; Mabus *et al.*, 1988; Isaacson *et al.*, 1989; Fried *et al.*, 1990). Fujino and Fried (1996) detected an increase in the number of goblet cells and crypt hyperplasia in *E. trivolvis*-infected hamsters. Although a single worm infection was sufficient to induce damage, wide variations in relation to the intensity of the infection were observed (Fried *et al.*, 1990). Isaacson *et al.* (1989) showed that hamsters with parasite burdens greater than 50 *E. caproni* adult worms developed unthriftiness and unkempt hair coat, weakness and unsteady gait, and produced loose stools. At necropsy, ballooning of the cecum was observed and the small intestine released large amounts of fluid and blood when opened.

Several studies have shown that the mouse model is suitable for the study of pathology in echinostome infections. Odaibo *et al.* (1988) and Hosier and Fried (1991) have shown that *E. caproni* infections in mice persist for more than 16 WPI in NMRI and ICR mice. In contrast, *E. trivolvis* is rejected from ICR mice at 4 WPI at the same infection doses. Comparative studies on the pathology of both species in mice have reported useful information. The histopathological effects of *E. caproni* in mice occur mainly in the surrounding area to the attachment sites of the worms. The intestine of mice infected with *E. caproni* showed a marked dilation in which the majority of the worms were found. Marked villous atrophy with fused and eroded villi and crypt hyperplasia were also seen (Bindseil and Christensen, 1984; Weinstein and Fried, 1991; Fujino and Fried, 1993a). The total mucosa thickness increased simultaneously with a hypertrophy of the tunica muscularis (Bindseil and Christensen, 1984). In the hyperplastic crypts, the mitotic rate was increased over that seen in normal crypts and the number of Paneth cells was reduced (Bindseil and Christensen, 1984; Fujino and Fried, 1993a). Cellular infiltration, consisting of lymphocytes, eosinophils, and plasma cells, was observed in the lamina propria and submucosa (Weinstein and Fried, 1991). An increase of collagen fibers and fibroblasts was also observed under the epithelium of the atrophic villi (Bindseil and Christensen, 1984; Fujino and Fried, 1993a). The non-dilated regions of the small intestine did not show differences with respect to non-infected controls. Simonsen *et al.* (1989) further examined the mucosal changes at the attachment sites

of *E. caproni* in NMRI mice. The sites consisted of a plug of grasped mucosa occupying the cavity of the ventral sucker. The study did not reveal any specific cellular response to the parasite.

The pathology of the related species *E. trivolvis* in mice show marked differences. No significant dilation of the gut and villous atrophy have been associated with *E. trivolvis* infections in mice (Weinstein and Fried, 1991; Fujino and Fried, 1993a; Fujino *et al.*, 1993). Differences in the enzymatic activity in the intestine of infected mice were also observed. The alkaline phosphatase activity in the microvilli of *E. caproni*-infected mice was significantly reduced with respect to non-infected controls, but less reduced in mice infected with *E. trivolvis* (Fujino and Fried, 1993a). Of particular interest are the differences observed in relation to the cell populations in the intestines of *E. caproni*- and *E. trivolvis*-infected mice. No significant increases in the number of Paneth cells, goblet cells, and mast cells were observed in *E. caproni* infections. In contrast, in *E. trivolvis* infections there was a marked increase of these cells (Bindseil and Christensen, 1984; Weinstein and Fried, 1991; Fujino and Fried, 1993a; Fujino *et al.*, 1993). One of the factors determining these differences may be that the effector mechanisms mediating the histopathological changes in *E. caproni* and *E. trivolvis* infections in mice are different. Bindseil and Christensen (1984) showed that pathological changes in *E. caproni*-infected mice were thymus-independent by comparing the results between conventional and congenitally athymic mice. In contrast, Fujino *et al.* (1993) showed that no increase in mast cells occurred in athymic mice and also the number of goblet cells was lower in athymic than in conventional *E. trivolvis*-infected mice. These facts suggested that thymus-dependent mechanisms operate in *E. trivolvis*-infected mice.

Little is known about the mechanisms inducing pathological alterations in echinostome infections. Simonsen *et al.* (1989) suggested that the mucosal changes in *E. caproni*-infected mice can be induced by the worm feeding activity, mechanical irritation, release of toxic substances, and or immune response to the parasite. It is well documented that *E. caproni* adults release large amounts of antigens (Thorndyke and Whitfield, 1987; Andresen *et al.*, 1989) that may affect the intestine of the host.

Extraintestinal infections with *E. trivolvis* in golden hamsters have been reported (Huffman *et al.*, 1988). The occurrence of *E. trivolvis* in extraintestinal sites results from worm crowding in heavy infections. The worms migrate to the common bile duct and can be found in the liver, gall bladder, and pancreas. The migration of the worms through the liver damages the blood vessels resulting in hemorrhage and mononuclear infiltration. Granulomas composed of giant cells were found in the liver surrounding the echinostome eggs. Secondary bacterial infections following the migration tract resulted in hepatic necrosis (Huffman *et al.*, 1986, 1988).

5.3. Immunology of the Infection

5.3.1. Manifestations of Resistance to Infection

There are multiple expressions of resistance to echinostome infections. One of them is the spontaneous expulsion of primary infections. Expulsion of echinostome adult worms from the host occurs with different dynamics depending on the model used. The elimination of *E. trivolvis* from mice occurs within 2–4 WPI (Weinstein and Fried, 1991), whereas in golden hamsters, adult worms survive for long periods of time (Mabus *et al.*, 1988). Similarly, *Echinostoma friedi* is able to survive for at least 12 WPI in hamster but the infection is expelled at 3–4 WPI in rats (*Rattus norvegicus*) (Toledo *et al.*, 2003a; Muñoz-Antoli *et al.*, 2004). *E. caproni* adults produce chronic infections in golden hamsters and mice but the worms are expelled in 6–8 weeks in rats (Odaibo *et al.*, 1988, 1989; Hansen *et al.*, 1991; Toledo *et al.*, 2004a). There are studies indicating that these responses are dependent on immunological factors. The survival of echinostome infections can be prolonged by immune-suppressive treatment or concurrent *Trypanosoma brucei* or *Schistosoma mansoni* infection (Christensen *et al.*, 1984, 1985; Fujino *et al.*, 1998a). Furthermore, the growth and development of echinostomes are affected by the host species and even the strain (Lee *et al.*, 2004; Toledo *et al.*, 2004a). Weaker antibody responses and higher seroantigen levels were observed in those host species in which the parasite was rejected

earlier (Toledo *et al.*, 2004b, 2005). Toledo *et al.* (2004b) associated the different levels of *E. caproni* seroantigens detected in hamsters and rats with different intestinal responses to the parasite that may be involved in the dynamics of worm rejection observed in each host species.

The generation of resistance to homologous and heterologous secondary infections is well documented. Sirag *et al.* (1980) developed models for studying the resistance to secondary infections in *E. caproni*-infected mice. Primary 20- or 28-day-old infections were sufficient to induce resistance to *E. caproni* challenge infections. Cross-protection against *E. caproni* infection was also induced by patent infection with *S. mansoni* and the resistance persists after antihelminthic treatment (Sirag *et al.*, 1980). Hosier *et al.* (1988) showed that ICR mice can develop resistance against homologous and heterologous challenge infection with either *E. caproni* or *E. trivolvis*. After homologous challenge, established worm burdens of *E. trivolvis* and *E. caproni* were 87.5 and 42%, respectively, with respect to those observed in primary infections. There was a 17% reduction in the worm burden of *E. caproni* superimposed upon a primary *E. trivolvis* infection and a 55% reduction of *E. trivolvis* challenge upon a primary infection with *E. caproni*.

There is evidence suggesting a role of the host immune response in the regulation of the fecundity of echinostomes. Differences in egg counts have been observed in *E. trivolvis* (Franco *et al.*, 1986), *E. caproni* (Toledo *et al.*, 2004a), and *E. friedi* (Muñoz-Antoli *et al.*, 2004) in relation to the host species. Muñoz-Antoli *et al.* (2004) noted that the egg output of *E. friedi* in rats was significantly lower than in hamsters though the worm burden was similar in both host species. Toledo *et al.* (2003a) showed that the egg output of *E. friedi* in hamsters decreased coinciding with the rise in the immune response. Lee *et al.* (2004) infected five strains of mice (C3H HeN, BALB c, C57BL6, FuB, and ICR) with *Echinostoma hortense*. Eggs were detected only in the feces of C3H HeN and ICR mice though worms developed in all of the mouse strains. The above-mentioned findings suggest that the genetic and immunological background of the host species are important determinants of parasite fecundity.

5.3.2. Effector Mechanisms of the Immune Response

A complex set of interacting and interrelated factors appears to govern the immune responses against echinostome infections. Furthermore, the response is different for each echinostome-host combination. In the intestine of infected hosts, *Echinostoma* spp. induce changes at the cellular level and in the expression of certain glycoconjugates that may be of importance in the regulatory response to worm populations.

Intestinal mastocytosis has been regarded as one factor responsible for intestinal helminth expulsion (Balic *et al.*, 2000). There are reports of conflicting data in relation to the role of mucosal mast cells in echinostome infections. In *E. trivolvis*-infected mice, the number of mast cells rapidly increased to reach a peak at approximately 2–3 WPI (Tani and Yoshimura, 1988; Fujino *et al.*, 1993, 1996a, 1998b), coinciding with worm expulsion. However, in athymic mice or mice treated with the immunosuppressive compound FK506, mastocytosis was suppressed but the kinetics of worm expulsion was similar to that of conventional and non-treated mice (Fujino *et al.*, 1993, 1998a). In contrast, Kim *et al.* (2000) detected that increases of mast cells in *E. hortense*-infected rats were concomitant with worm expulsion. Similarly, it is not clear if increased eosinophil numbers are concerned with worm expulsion in echinostome infections. Eosinophilic infiltration is a characteristic feature in the intestinal mucosa of mice infected with *E. caproni* (Bindseil and Christensen, 1984) and *E. trivolvis* (Fujino *et al.*, 1996a). Hansen *et al.* (1991) demonstrated increased levels of peripheral eosinophilia in rats infected with *E. caproni*. The degree of peripheral eosinophilia was closely related to the worm burden, suggesting a potential role in the regulatory response against *E. caproni* in rats. Further studies are needed to clarify this point.

Mice have served as models to determine that increased mucus production associated with goblet cell hyperplasia are involved in worm expulsion in echinostome infections. In mice, *E. caproni* induced chronic infections concomitantly with reduced goblet cell numbers (Bindseil and Christensen, 1984; Weinstein and Fried, 1991; Fujino and Fried, 1993a). In contrast, in *E. trivolvis*-infected mice, the goblet cell numbers increased coinciding with worm expulsion

(Fujino *et al.*, 1993). Expulsion was delayed when mice were treated with dexamethasone. In contrast to non-treated control mice, dexamethasone-treated mice did not show an increase in their goblet cell numbers. Finally, worm expulsion corresponded with the cessation of dexamethasone administration and an increase in the goblet cell numbers. (Fujino *et al.*, 1996b, 1997). When mice were treated with dexamethasone, worm expulsion was delayed accordingly. As in normal mice, dexamethasone administration to immunodeficient SCID mice delayed the expulsion of *E. trivolvis* (Fujino *et al.*, 1998b). Fujino *et al.* (1996b) demonstrated that primary infection in mice with *E. trivolvis* generated resistance to challenge infections with *E. caproni*. Fujino *et al.* (1996c) showed that primary infection with the nematode *Nippostrongylus brasiliensis* in C3H HeN mice induce a rapid expulsion of challenge infections with either *E. trivolvis* or *E. caproni*. Increased secretion of mucus by hyperplastic goblet cells associated with primary infections was suggested to be responsible for the resistance in both cases. Fujino and Fried (1993b) studied the lectin labeling patterns in the small intestine of C3H mice infected with either *E. caproni* or *E. trivolvis*. They noted marked differences in the distribution and intensity of glycoconjugates in infected hosts. In *E. trivolvis*-infected mice, the amount of mucins, including *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine, were strongly expressed in association with an increased number of goblet cells. In *E. caproni*-infected mice, the binding of most lectins was reduced in accord with the loss of goblet cells. Interestingly, *E. trivolvis*-infected hamsters did not show a marked increase of goblet cell numbers or intensity of lectin labeling and chronic infections developed (Fujino and Fried, 1996).

Although the involvement of goblet cells in the expulsion of echinostome infections seems evident, Frazer *et al.* (1999) showed that the situation is more complex than previously expected and observed a marked goblet cell response in RAG-2-deficient mice. However, worms survived as they did in ICR mice. This suggests that other immune mechanisms are also involved in worm expulsion. In this sense, Fujino *et al.* (1994) had previously shown that factors present in mouse serum induced retraction of collar spines in *E. trivolvis*, probably enhancing worm expulsion.

The generation of systemic antibody responses in echinostome infections depends on the host–parasite combination. *E. caproni* induces rapid and strong responses in mice (Agger *et al.*, 1993; Graczyk and Fried, 1994; Toledo *et al.*, 2005) and strong, but slower, responses in hamsters (Simonsen *et al.*, 1991; Toledo *et al.*, 2004b). In rats, the responses are weak (Toledo *et al.*, 2003b, 2004c). *E. trivolvis* elicits strong responses in mice (Graczyk and Fried, 1995) but weaker responses in hamsters (Mabus *et al.*, 1988). The influence of the IgG systemic response in the course of the infections is not known but, interestingly, in *E. caproni* infections, strong responses have only been observed in those host species in which the parasite induces chronic infections. (Toledo *et al.*, 2004b, 2005). This suggests that these responses may be a collateral consequence of the infection without significant influence on the course of the infection. Toledo *et al.* (2004b) suggested that the different systemic antibody responses to *E. caproni* observed in each host species are related to differences in the local inflammatory responses. Juvenile and adult worms secrete antigens that can cross the intestinal mucosa, reach the circulatory system, and induce systemic antibody responses by B-cell stimulation. It is known that the passage of antigens through the intestinal mucosa is mediated by local inflammation (Yu and Perdue, 2001; Avila *et al.*, 2003). Thus, differences in mucosal inflammatory responses may result in differences in antibody responses. In this sense, high levels of *E. caproni* seroantigens have been detected concomitantly with high systemic antibody responses in hamsters and mice, whereas low levels of seroantigens and antibody responses were detected in rats (Toledo *et al.*, 2004b, 2005).

Agger *et al.* (1993) detected IgM, IgG, and IgA responses in the intestine of *E. caproni*-infected NMRI mice. However, only IgA antibodies were measured at significant levels in the intestinal lumen after 28 DPI. Simonsen and Andersen (1986) demonstrated antibodies to the surface of *E. caproni* in the serum from infected mice and showed that worms obtained from immune mice were covered by antibodies. These immune complexes could activate antibody- and complement-dependent cellular cytotoxicity (Simonsen and Andersen, 1986; Simonsen *et al.*, 1990).

The cytokine profiles during echinostome infections have been poorly studied. Brunet *et al.* (2000) showed that the establishment of

E. caproni chronic infections in mice is biased toward a Th1 phenotype and characterized by antigen-specific IgG2a antibody production and elevated levels of IFN- γ . To assess the role of IFN- γ , Brunet *et al.* (2000) injected anti-IFN- γ antibody to *E. caproni*-infected mice and found a significant reduction in worm establishment, suggesting an important role of IFN- γ in the development of chronic infections. This confirms the fact that development of chronic infections could be associated with high local inflammatory responses (Toledo *et al.*, 2004b).

5.3.3. *Evasion of the Host Immune Responses*

Although echinostomes induce significant immune responses in their hosts, in most cases, chronic infections are developed. In this sense, several mechanisms to evade the host immune response have been suggested for *E. caproni* infections. It has been observed that antibodies which are bound to the surface of *E. caproni* are rapidly lost *in vivo* (Simonsen and Andersen, 1986; Simonsen *et al.*, 1990). Furthermore, Andresen *et al.* (1989) showed that newly excysted juvenile and adult worms of *E. caproni* shed surface antigens and the turnover rate was very high (8–15 min). This was regarded as an adaptation to withstand the host immune response by rendering the worms inaccessible to the immune attack (Andresen *et al.*, 1989). Toledo *et al.* (2004c) showed that the antigenic composition of *E. caproni* may change over time which may serve to avoid the specific immune attack.

5.3.4. *Antigenic Characterization*

There is little information about the antigenic composition of echinostomes. Andresen *et al.* (1989) suggested that the surface antigens of *E. caproni* were mainly responsible for the induction of antibody responses in mice. SDS-PAGE and Western blot analysis of *in vitro* shed and detergent-solubilized antigens indicated that the four major antigens released from adult worms had molecular weights of 26, 66, 78, and 88 kDa. However, the composition of the excretory–secretory products of *E. caproni* appears to be strain-dependent. Trouve and Coustau (1998) compared the excretory–secretory products of three

isolates of *E. caproni* (Madagascar, Egypt, and Cameroon). Although polypeptide patterns from these isolates shared most major bands, isolated specific differences could be detected. Toledo *et al.* (2004c) characterized the somatic and excretory-secretory antigens of *E. caproni* from rats. Analysis by SDS-PAGE showed some common as well as unique polypeptides for each type of extract. Toledo *et al.* (2004c) also defined by Western blot 11 and 7 major antigenic polypeptides in the somatic and excretory-secretory products, respectively, which were recognized by sera from experimentally infected rats.

The role of echinostome antigens in the course of the infection remains unknown. Thorndyke and Whitfield (1987) suggested that *E. caproni* secretes vasoactive polypeptide-like components from the tegument that may affect the host intestinal physiology. Trouve and Coustau (1998) suggested that excretory-secretory products are involved in gregarious behavior or pairing. Toledo *et al.* (2004c) demonstrated that some polypeptides are specifically expressed in the juvenile phase of *E. caproni* suggesting a role in the host-parasite relationships. Marcilla *et al.* (2004) demonstrated an increase in protein tyrosine phosphorylation in *E. friedi* adult worm extracts in the presence of bile and also suggested the presence of distinct tyrosine phosphorylated epitopes being recognized by distinct antiphosphotyrosine antibodies. This could be a parasite mechanism of response to the host environment facilitating worm establishment.

5.4. Immunodiagnosis

Immunodiagnostic tests based on indirect ELISA have been developed to detect anti-*E. caproni*, anti-*E. trivolvis* and anti-*E. friedi* serum IgG (Simonsen *et al.*, 1991; Agger *et al.*, 1993; Graczyk and Fried, 1994, 1995; Toledo *et al.*, 2003a, 2004a, b, 2005). Using adult crude worm and excretory-secretory antigens, it was possible to detect anti-*E. caproni* IgG in serum of mice from 2 WPI (Agger *et al.*, 1993; Toledo *et al.*, 2005). With glycocalyx membrane of adult worms of *E. caproni*, it was possible to detect IgG in mice by 8 DPI (Graczyk and Fried, 1994). In hamsters and rats, anti-*E. caproni* responses were detected from 6–8 WPI (Simonsen *et al.*, 1991; Toledo *et al.*, 2004a).

Anti-*E. trivolvis* IgG were detected in mice from 10 DPI, though reciprocal cross-reactivity with *E. caproni* was observed (Graczyk and Fried, 1995). However, a major limitation of these methods is that antibody response indicates previous exposures rather than active infections (Toledo *et al.*, 2003b).

The development of a coproantigen capture ELISA has provided a rapid and sensitive method for the diagnosis of echinostome infections. Coproantigens of *E. caproni* were detected earlier (1–2 DPI) than antibodies in hamsters and rats. Moreover, coproantigen levels reverted to negative values coinciding with worm expulsion, allowing to distinguish between past and active infections (Toledo *et al.*, 2003b, 2004b).

5.5. Human Infections

Haaseb and Eveland (2000) reported that about 20 species belonging to eight genera of Echinostomatidae have been found parasitizing humans. Although echinostomiasis occurs worldwide, most human infections have been reported from foci in East and Southeast Asia (Graczyk and Fried 1998; Chai and Lee, 2002). Human morbidity is due to the prolonged latent phase, asymptomatic presentations, and similarity of symptoms to other intestinal helminthiasis (Graczyk and Fried, 1998). Clinical symptoms depend on the parasite load and have been recently reviewed by Fried *et al.* (2004). Heavy infections are associated with eosinophilia, abdominal pain, watery diarrhea, anemia, edema, and anorexia (Chattopadhyay *et al.*, 1990). Pathological damage includes catarrhal inflammation, erosion and even ulceration of the duodenum (Chai *et al.*, 1994a; Cho *et al.*, 2003). There are no studies on the immunology of human echinostome infections.

6. THE FAMILY GYMNOPHALLIDAE

6.1. Background

This family consists of a small group of digeneans occurring in the intestine, gall bladder, and bursa Fabricii of birds and also in the

intestine of mammals. The taxonomy of the family, inclusive of generic classification, is unsatisfactory due to considerable homogeneity of its members, their small body size, and difficulty in observing the internal structures of these digeneans (Scholz, 2002). Although the number of genera included within this family varies greatly (Ching, 1995; Lee and Chai, 2001), a recent revision of the family accepts a total of five valid genera (*Gymnophalloides*, *Parvatrema*, *Gymnophallus*, *Pseudogymnophallus*, and *Bartolius*) (Scholz, 2002). A typical gymnophallid life cycle involves bivalves as first intermediate host, and bivalves, polychaetes, gastropods, or brachiopods as second intermediate hosts. The definitive host becomes infected after ingestion of the second intermediate host harboring the metacercariae (Ching, 1995).

Within the Gymnophallidae, studies on the pathology and immunology of the infection are available only for one species, *Gymnophalloides seoi* (Figure 9). This is a minute intestinal fluke that has been reported from humans in Korea (Chai and Lee, 2002; Chai *et al.*, 2003). The first intermediate host of *G. seoi* is unknown, but a second intermediate host is the oyster *Crassostrea gigas*. Humans, the oystercatcher (*Haematopus ostralegus*), and wading birds are natural definitive hosts (Lee and Chai, 2001). At the experimental level, several avian species, gerbils, hamsters, cats, and several strains of mice are susceptible laboratory hosts (Lee *et al.*, 1997b; Ryang *et al.*, 2001). Mice have been used as experimental hosts in most of the studies relative to the pathology and immunology of *G. seoi* infections. In mice, the worms inhabit the small intestine but are usually rejected within 3 WPI (Lee and Chai, 2001).

6.2. Pathology of the Infection

The pathology of *G. seoi* infection varies depending on the host species. The mucosal damage in C3H HeN mice is not severe (Chai *et al.*, 2001). In contrast, the pathology caused in humans appears to be considerably greater. Although in endemic areas the degree of symptoms in humans is variable in individual patients, some cases of severe gastroenteritis and signs of acute pancreatitis have been reported (Lee *et al.*, 1993a; Lee and Chai, 2001; Chai *et al.*, 2003).

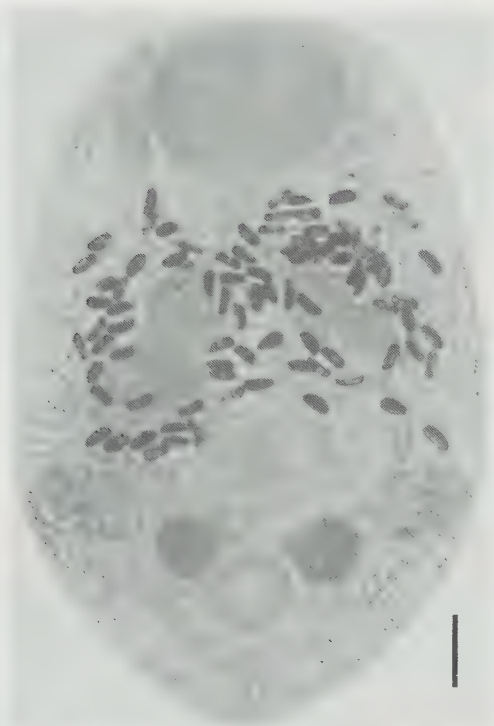


Figure 9 *Gynophalloides seoi*: An adult worm from a naturally infected human; the worm was stained in acetocarmine. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 50 μ m.

Detailed studies on the pathology of *G. seoi* infections have been made in immunocompetent and immunosuppressed mice C3H, HeN mice (Chai *et al.*, 2001). In immunocompetent mice, relatively few worms were seen; worms were found pinching and grasping the host epithelial layer with their oral suckers. The histopathology was characterized by villous atrophy and crypt hyperplasia, with inflammatory reactions in the villous stroma and the crypt (Figure 10). The most striking observation was, however, a marked goblet cell hyperplasia along the villous epithelia. The goblet cell hyperplasia was greater in the jejunum than in the duodenum or ileum (Chai *et al.*, 2001). However, the severity of these features appeared to be less than in other intestinal trematode infections. The pathological changes were almost resolved at 14-21 DPI and only some pathological signs

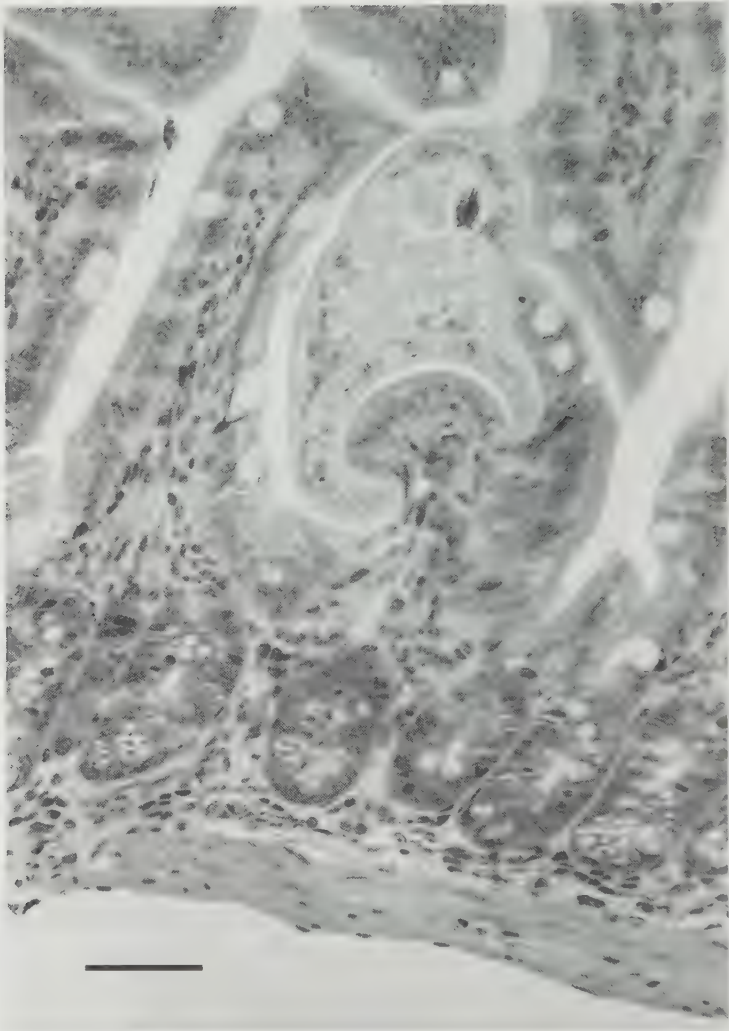


Figure 10 Small intestine of a mouse experimentally infected with *Gymnophalloides seoi* and necropsied at 5 DPI; the preparation was stained with hematoxylin and eosin. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 100 μm .

remained focally at the end of the experiment at 21 DPI (Chai *et al.*, 2001). In general, the histopathology of *G. seoi* infections in immunosuppressed C3H/HeN mice was similar to that described for immunocompetent mice. However, the destruction of the villi adjacent to

the worms was more severe than in immunocompetent mice. Moreover, no remarkable hyperplasia was observed in immunosuppressed mice (Chai *et al.*, 2001).

The reason for the mild histopathology of *G. seoi* infections in mice remains unknown. Chai *et al.* (2001) suggested that this could be due to the small number of worms actually parasitizing the intestine of mice. In fact, the recovery rate of *G. seoi* in C3H/HeN mice was 11.8% (Lee *et al.*, 1997b). With regard to mechanisms eliciting pathogenicity in *G. seoi* infections, mechanical irritation by the flukes was considered an important mechanism. Chai *et al.* (2001) noted that the nearby villi were pressure-atrophied and even severely destroyed, with some areas showing a complete loss of villi. Moreover, cysteine proteases have been isolated from metacercariae and adults of *G. seoi*. However, these proteases appear to be implicated more in nutrient uptake and evasion of the host immune response than in eliciting histopathology to the host (Choi *et al.*, 1998a, b).

6.3. Immunology of the Infection

Resistance to *G. seoi* infection is manifested by differences in worm recovery rates and worm growth depending on the host species and even in differences between strains of hosts. Moreover, spontaneous expulsion after temporary establishment occurs in most of the experimental models studied (Lee *et al.*, 1997b). *G. seoi* is able to establish in several species of avian and mammal hosts, though differences between host species can be found in parameters such as worm establishment, worm measurements, fecundity, or life span (Lee *et al.*, 1997b; Chai *et al.*, 1999; Ryang *et al.*, 2001). In mice, *G. seoi* infections are spontaneously expelled within 3 WPI but the course of the infection differs depending on the strain of mice (Lee *et al.*, 1997b; Chai *et al.*, 1999) indicating a genetic background in the resistance to infection. From a total of seven strains of mice (KK, C3H HeN, ICR, BALBc, ddY, A, and C57BL/6), C3H/HeN seems to be the most susceptible in terms of greater worm recovery rates, worm growth, and uterine egg counts. In contrast, C57BL 6

mice rejected the worms more rapidly than the other strains (Lee *et al.*, 1997b; Seo *et al.*, 2003). Comparative studies on the development of *G. seoi* in immunocompetent and immunosuppressed mice indicated that the differential susceptibility is dependent on the host immune response. Immunosuppression of C3H HeN mice with prednisolone resulted in higher worm recovery rates and growth of the worms than in the immunocompetent controls (Lee *et al.*, 1997b; Chai *et al.*, 1999). Furthermore, the immunosuppression also enhanced the fecundity of the worms since worms recovered from immunosuppressed mice had significantly more uterine eggs than those from controls (Chai *et al.*, 1999). Similarly, the survival of *G. seoi* adults in C57BL 6 mice was increased after treatment of the mice with prednisolone (Seo *et al.*, 2003).

Little is known about the immune effector mechanisms responsible for the short-term parasitism of *G. seoi* observed in mice. However, the observation of a strong proliferation of goblet cells previously to the worm expulsion in *G. seoi*-infected mice suggests that this can be one of the effector mechanisms involved in worm rejection (Chai *et al.*, 2001). Strong goblet cell responses were observed at 3–7 DPI in immunocompetent C3H HeN mice and most of the worms were expelled at 7–14 DPI. In immunosuppressed C3H HeN mice, only relatively low levels of goblet cell hyperplasia were observed concomitantly with a higher survival of the worms (Chai *et al.*, 2001). Seo *et al.* (2003) studied the goblet cell hyperplasia and the secretion of mucus in relation to worm recovery in immunocompetent and immunosuppressed C56BL 6 mice. A significant reduction in goblet cell hyperplasia and mucins together with a greater worm recovery rate was observed in immunosuppressed mice with respect to immunocompetent mice. Although these facts suggest the involvement of goblet cells in the expulsion of *G. seoi* infections, further studies are required relative to the participation of other immune mechanisms.

Parasites can protect themselves from host immune responses by a variety of strategies (Maizels *et al.*, 1993). Immunological modulation strategies include the direct or indirect blocking by parasites on the effect of antibodies. This may involve the use of proteases capable of degrading host immunoglobins. Cleavage of immunoglobins by

parasite proteases has been demonstrated in trematodes such as *S. mansoni* (Auriat *et al.*, 1981) or *Fasciola hepatica* (Berasain *et al.*, 2000). Choi *et al.* (1998a) purified a 16-kDa cysteine protease from metacercariae of *G. seoi* that showed the ability to degrade human immunoglobulins. The protease only partially degraded IgG2a after incubation overnight. However, heavy chains of IgA were completely degraded and a significant degradation of light chains was also observed. The IgA-cleaving activity of this cysteine protease suggests that *G. seoi* can evade some of the host effector immune mechanisms, especially mucosal immunity, in the intestine of the host.

With regard to the antigenic composition of *G. seoi*, the studies are limited to the characterization of two cysteine proteases. As mentioned in the above paragraph, Choi *et al.* (1998a) identified a 16-kDa cysteine protease from *G. seoi* metacercariae. Apart from the IgA-cleaving activity, the protease was able to degrade extracellular matrix proteins such as collagen or fibronectin, but only slight digestion of hemoglobin was observed. Choi *et al.* (1998a) suggested that this protein may also be involved in the nutrition of *G. seoi* by degrading extracellular matrix proteins. ELISA and Western blot analysis demonstrated that this protease is not antigenic in the definitive host. Another cysteine protease, weighing 40 kDa, was purified from adult crude extracts of *G. seoi* by Choi *et al.* (1998b). The enzymatic activity was similar to that of 16-kDa protease, but did not show the ability to degrade human immunoglobulins.

6.4. Human Infections

Human infections with *G. seoi* have only been recorded in Korea (Chai and Lee, 2002; Chai *et al.*, 2003; Fried *et al.*, 2004). *G. seoi* was first discovered in a woman who suffered acute pancreatitis and gastrointestinal discomfort (Lee *et al.*, 1993a). *G. seoi* is highly prevalent among villagers in the southwestern coastal islands of Korea, where half of the population was infected (Chai *et al.*, 2000b). The people became infected by consuming raw oysters (Lee and Chai, 2001). Clinical symptoms of this infection include loose stools, pancreatitis, indigestion, diarrhea, and gastrointestinal discomfort (Lee and Chai,

2001; Chai and Lee, 2002). *G. seoi* infections may require medical attention because of their relationships with pancreatic diseases (Lee *et al.*, 1995; Lee and Chai, 2001). There is no literature available on the pathology or immunology of *G. seoi* human infections.

7. THE FAMILY HETEROPHYIDAE

7.1. Background

The family Heterophyidae contains small egg-shaped trematodes with infective metacercariae that are usually encysted in fish second intermediate host. A striking feature of the members of this family is the possession of a gonotyle or genital sucker (Yamaguti, 1971). This author accepted 13 subfamilies within the family which may be differentiated on the basis of body morphology, presence of a circumoral crown of spines, extension of the vitellaria and uterus, morphology of the testes, and location of the genital pore.

The definitive host becomes infected by eating raw or poorly cooked fish harboring metacercariae. Heterophyids show little specificity toward the definitive host and numerous fish-eating mammals, including humans, can be infected. The adult worms live between the villi of the anterior region of the small intestine and release fully embryonated eggs into water. The eggs are then ingested often by littorine snails (particularly *Littorina littorea* and *L. scutulata*), and hatch within the snail's intestine. Intramolluscan development comprises sporocyst and redial stages and cercariae are released into the water where they typically penetrate shore-fish, such as cunners, gudgeon, and charr, and encyst on the surface of the fish. Metacercariae may remain viable for years (Fried *et al.*, 2004).

Although there is a great number of genera within the Heterophyidae (Yamaguti, 1971), most of the studies in relation to the pathology and immunology of these infections are focused on *Metagonimus yokogawai* (Figure 11). This species is parasitic in humans in Asia (Chai and Lee, 2002) and its life cycle can be maintained easily in the laboratory in various experimental hosts, thus facilitating studies on heterophyids.

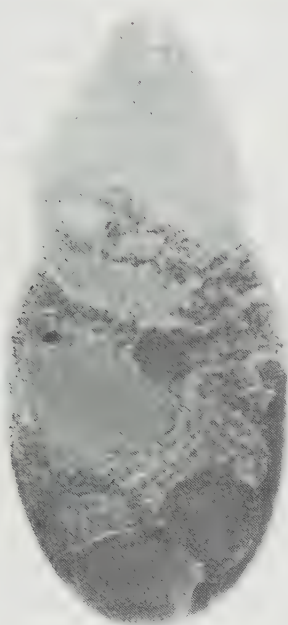


Figure 11 An adult worm of *Metagonimus yokogawai* from an experimentally infected dog necropsied at 14 DPI; the worm was stained in acetocarmine. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 150 μ m.

7.2. Pathology of the Infection

In general, the clinical symptoms due to heterophyid infections are mild and transient, unless hosts are heavily infected or immunocompromised (Chai and Lee, 2002). The most prominent symptoms are malabsorption and diarrhea (Lee *et al.*, 1981; Kang *et al.*, 1983; Cho *et al.*, 1985; Snyder *et al.*, 1989; Hong *et al.*, 1990; Yu *et al.*, 1997). The pathological features of *M. yokogawai* infections have been studied in several hosts, including cats, dogs, rats, and humans. Although mice are not suitable hosts to *M. yokogawai*, because most worms are rapidly expelled within the first few DPI (Chai *et al.*, 1984, 1995), they have been used to analyze several pathological features during the early phases of the infection (Hong *et al.*, 1990; Chai *et al.*, 1995; Yu *et al.*, 1997).

The adult worms of *M. yokogawai* parasitize mainly the duodenum and proximal jejunum (Figure 12), though in heavy infections the location of the worms tends to extend more distally (Lee *et al.*, 1981). In early stages of the infection, the worms are located in the glandular lumens of the crypts of Lieberkühn, whereas in the later phases they are found between the villi (Jang *et al.*, 1985; Chai *et al.*, 1995). The gross pathology elicited by *M. yokogawai* is similar in all host species studied. The main pathological features in the early phases of infection are villous atrophy, characterized by fusion, thickening, and shortening of the villi, hypertrophy of the crypts of Lieberkühn, resulting in a decrease of the villous/crypt ratio, enlargement of mesenteric lymph nodes, and inflammatory responses with cell infiltration (Chai *et al.*, 1994b, 1995; Lee *et al.*, 1981; Kang *et al.*, 1983; Rho *et al.*, 1984; Hong *et al.*, 1997; Yu *et al.*, 1997). However, these mucosal changes are rapidly restored, although the adult worms remain in the intestine. In cats, the mucosal regeneration began at 4 WPI, and the mucosal architecture was normal at 8 WPI (Lee *et al.*, 1981).

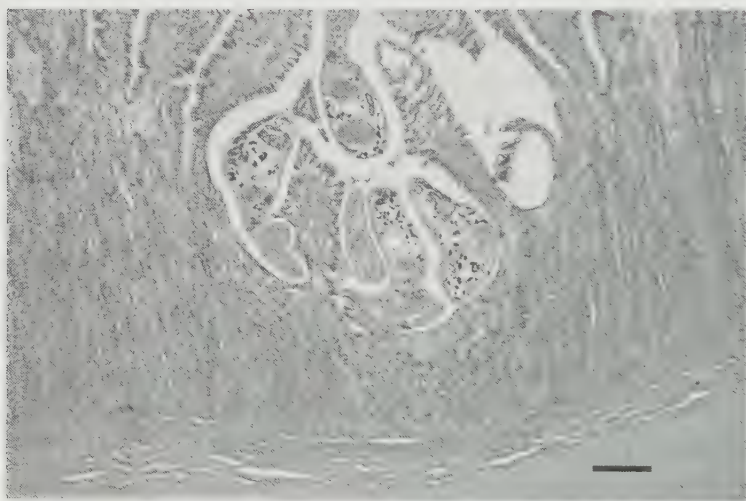


Figure 12 Small intestine of a dog experimentally infected with *Metagonimus yokogawai* and necropsied at 7 DPI; the preparation was stained with hematoxylin and eosin. Photomicrograph courtesy of Jong-Yil Chai. Scale bar: 200 μ m.

The factors determining villous atrophy in metagonimiasis are not well understood. One mechanism involved may be mechanical pressure and villi destruction by the worms. Apparently, the lining of the epithelium of the villi near the worms was compressed and destroyed and also an inflammatory response in the adjacent lamina propria was observed (Lee *et al.*, 1981). In this context, the circumoral spines in the related species *Centrocestus armatus* was thought to cause mechanical damage to the epithelial cells of the intervillous crypts resulting in fusion of the villi adjacent to the worms (Hong *et al.*, 1997). Although mechanical damage can be a cause of villous atrophy in metagonimiasis, other factors appear to be involved. Lee *et al.* (1981) observed that the intestinal mucosa of *M. yokogawai*-infected cats was completely restored at 8 WPI, at which time most of the adult worms remained viable in the intestine. Chai (1979) suggested that bulky foamy intestinal content and intraluminal pressure by gas and mucous content caused villous atrophy in *M. yokogawai*-infected rats. However, the role of these aforementioned factors appeared to be minimal in cats (Lee *et al.*, 1981). Chai *et al.* (1994b) suggested that pathology induced by *M. yokogawai* in rats may be related to changes in the number and location of intraepithelial lymphocytes. In the *M. yokogawai*-infected rats, the number of intraepithelial lymphocytes was increased with respect to the controls and also the intraepithelial lymphocytes have moved from the basal region of the epithelium to the intermediate or apical region. Yu *et al.* (1997) proposed that the villous atrophy was caused by a decrease in cell proliferation induced by *M. yokogawai*. Lower levels of proliferating cell nuclear antigen were observed in *M. yokogawai*-infected ICR mice with respect to controls. Because decreased levels of proliferating cell nuclear antigen indicate reduction in the population of proliferative cells, Yu *et al.* (1997) suggested that *M. yokogawai* induces inhibition of cell proliferation in the intestinal crypts in the early stages of the infection.

Presumably, the villous atrophy is the main cause of the malabsorption and watery diarrhea that characterize metagonimiasis. The distribution of watery content along the intestine of dogs experimentally infected with *M. yokogawai* suggested that it was the product of poor digestion due to villous atrophy (Cho *et al.*, 1985). This is

supported by the changes observed in the brush border enzyme activities in *M. yokogawai*-infected mice (Hong *et al.*, 1990). The nature of these changes was different in each intestinal segment. The activities of disaccharidases, alkaline phosphatase, and L-leucine aminopeptidase were decreased in the duodenum and proximal jejunum, but increased in the distal jejunum. The reduced activity in the areas which constitute the main habitat of the parasite was explained by the damage of the enterocytes at the upper layer of the crypts and the reduction of the mucosal surface area due to villous changes. In contrast, the greater activity in the distal jejunum could be a compensatory mechanism due to increased concentrations of substrates in relation to the malabsorption in the more proximal areas of the small intestine (Hong *et al.*, 1990).

Little is known about the cellular response at the intestinal level in heterophyid infections. Inflammatory cell infiltration is a common feature in infections with *M. yokogawai* (Lee *et al.*, 1981; Kang *et al.*, 1983; Chai *et al.*, 1995; Hong *et al.*, 1997). In dogs, the cell infiltration was mainly composed of eosinophils, lymphocytes, and plasma cells (Kang *et al.*, 1983), whereas neutrophils were the main cellular component in mice (Chai *et al.*, 1995). Interestingly, the cell infiltration remained after the restoration of the villi in *M. yokogawai*-infected cats (Lee *et al.*, 1981). Goblet cell responses in heterophyid infections have been poorly studied. Chai (1979) observed that the number of goblet cells was increased in *M. yokogawai*-infected albino rats. In contrast, a significant depletion of goblet cells was observed in the small intestine of cats and dogs experimentally infected with *M. yokogawai* (Lee *et al.*, 1981; Kang *et al.*, 1983). It was suggested that the depletion in goblet cell number could be related to mechanical irritation induced by the worms (Lee *et al.*, 1981). However, other factors could be involved since the depletion was not restricted to the area occupied by the worms but extended to the entire small intestine. The mucosal mast cell response only has been studied in *M. yokogawai*-infected Sprague-Dawley rats (Chai *et al.*, 1993). The maximal mucosal mast cell response was observed at 3 WPI coinciding with a significant decrease in the worm recovery rate.

7.3. Immunology of the Infection

7.3.1. *Manifestations of Resistance to Infection*

Immunity against heterophyid infections can be manifested in different ways. Several studies have demonstrated the development of cellular and humoral responses to these infections (Kang *et al.*, 1983; Abou-Zakham *et al.*, 1987; Cho *et al.*, 1987; El-Ganayni *et al.*, 1989; El-Ridi *et al.*, 1990; Ditrich *et al.*, 1991; Chai *et al.*, 1993; Lee *et al.*, 1993b; Hong *et al.*, 1997). The development of acquired immunity to *M. yokogawai* infections has been suggested. Kang *et al.* (1983) compared worm recovery rates and pathological changes induced in dogs in primary infections and reinfections. The worm recovery rate was lower in reinfected than in primary infected dogs. Moreover, the reinfected dogs showed less severe pathology and the damage was shifted to more distal regions of the intestine than in primary infections. The restoration of the mucosa was also more rapid in the reinfected dogs. However, the mechanisms involved in this immunity were not explored further.

Further evidence of immune-mediated resistance to infection with *M. yokogawai* was obtained by immunosuppression of ICR mice with prednisolone (Chai *et al.*, 1984). Immunosuppression significantly enhanced worm survival. Chai *et al.* (1984) reported that in immunocompetent mice, the worm recovery rates decreased from 66 (at 1 DPI) to 0.1% (at 4 DPI). In contrast, the worm recovery rates in treated mice ranged from 16 to 80% up to 21 DPI. The location of the worms was also different between both groups of mice. In immunosuppressed mice, most of the adult worms were found in the jejunum, whereas in the untreated mice the worms appeared more distally and almost 50% were located in the ileum from 1 DPI.

Chai *et al.* (1995) showed that the pathological features of *M. yokogawai* infection were different in prednisolone-treated and untreated ICR mice. Immunosuppression induced more severe mucosal damage and allowed deeper invasion of the worms into the submucosa of the small intestine. A marked degeneration and loss of the mucosal layers in the duodenum and jejunum was observed at 5 DPI.

Concomitantly, the worms were located deep in the submucosa facing the muscular layer. Interestingly, the mucosal damage was gradually recovered at 7-21 DPI, despite the continuous immunosuppression. In this context, it should be noted that immunosuppression has been regarded as a factor in explaining the extraintestinal locations observed in some heterophyid infections (Chai and Lee, 2002). In immunocompromised patients, eggs can enter the bloodstream and cause ectopic parasitism.

There are some evidences of a genetic basis of resistance to *M. yokogawai* infections. Chai *et al.* (1984) studied susceptibility of this infection in four strains of mice (CBH, C57BL, DBA, and KK) and the greatest worm recovery rates were observed in C57BL and KK mice. Moreover, the worms were larger and were located more proximally in the small intestine in these two strains of mice than in the other strains. Guk *et al.* (2005) studied the susceptibility of inbred mouse strains to *M. yokogawai*, *M. miyatai*, and *M. takahashii* infections using BALB_c, ddY, C57BL 6J, C3H HeN, and A J mice, with H-2 haplotypes d, s, b, k, and a, respectively. The results showed that susceptibility of mice to *Metastrongylus* infection varied according to mouse strain and parasite species but is suggested to be independent of the mouse H-2 haplotype.

7.3.2. Effector Mechanisms of the Immune Response

Little is known about the effector mechanisms involved in the immune response against heterophyid infections and intensive studies are required to understand the role of cellular and humoral responses in the host defenses against such infections. Kang *et al.* (1983) suggested that pathological changes induced by *M. yokogawai* in experimentally infected dogs could be involved in worm expulsion. However, the particular mechanisms that could be implicated in worm expulsion were not studied. Some types of cells have been suggested to be related to the mechanisms determining the rejection of *M. yokogawai* adult worms. However, no functional studies have been performed and the relationships were based only on the fact that cell counts increased coinciding with worm expulsion. Mucosal mast

cells progressively increased during the first days of the *M. yokogawai* infection in Sprague-Dawley rats reaching a maximum concomitantly with an abrupt decrease in the worm recovery rate (Chai *et al.*, 1993). It was suggested that mast cells may induce mucosal changes facilitating the worm expulsion. Intraepithelial lymphocytes were also suggested as one of the effector immune mechanisms in the *M. yokogawai* infections since changes in number and location of these cells were observed in *M. yokogawai*-infected rats (Chai *et al.*, 1994b).

The humoral response in heterophyid infections has been poorly studied, though it is known that both systemic and local antibody responses occur in these infections. The specific IgG levels in the serum of *M. yokogawai*-infected cats were studied by Cho *et al.* (1987) using metacercarial and adult crude extract as antigen. Indirect ELISA analysis showed that IgG levels began to rise from 7 DPI and the maximum levels were observed at 2–4 WPI with both antigens. Thereafter, the antibody levels decreased. Elevated levels of IgG, IgM, and IgE have been detected in the serum of humans infected with *Heterophyes heterophyes* (El-Ganayni *et al.*, 1989; Martínez-Alonso *et al.*, 1999; Pica *et al.*, 2003). In the intestine, the levels of IgG, IgM, and IgA were increased (El-Ganayni *et al.*, 1989). Systemic IgG response has also been detected in humans infected with *Haplorchis taichui* and *M. yokogawai*, respectively (Ditrich *et al.*, 1991; Lee *et al.*, 1993b). There is some controversy on the potential role on the antibody response in the course of heterophyid infections. An inverse relationship between the antibody levels and the load of the infection has been often observed, suggesting that humoral response may be involved in worm expulsion (El-Ganayni *et al.*, 1989; Ditrich *et al.*, 1991). In contrast to these findings, Cho *et al.* (1987) detected that specific IgG levels were directly related to the number of *M. yokogawai* adult worms in experimentally infected cats.

7.3.3. Antigenic Characterization

Studies on the antigenicity of heterophyid worms have been done mainly on *M. yokogawai*. Immunogold labeling studies using sera

from cats experimentally infected with *M. yokogawai* have shown that antigenic materials were concentrated in the tegumental syncytium as well as the cytoplasm of tegumental cells and the epithelial lamella of the cecum (Ahn *et al.*, 1991; Rim *et al.*, 1992). Lee *et al.* (1993b) studied the antigenic fractions of metacercariae of *M. yokogawai*. A total of 14 protein bands, ranging from 8 to 200 kDa, were detected by SDS-PAGE. Of these bands, 11 reacted with sera of *M. yokogawai*-infected cats and humans in the Western blot analysis. However, cross-reactivity with other trematodes is usual in heterophyid infections (Hassan *et al.*, 1989; Lee *et al.*, 1993b; Kim, 1998) and most of the bands detected by Lee *et al.* (1993b) were also recognized by other trematodiasis sera. The protein bands at 22 and 66 kDa were the most specific.

7.4. Immunodiagnosis

Although heterophyids generate detectable immune responses, there are relatively few studies concerning the immunodiagnosis of these infections. One of the reasons for the lack of research may be the difficulty in preparing antigens because of the small size of the adult worms. Although this is a fact, some studies on indirect ELISA and Western blot methods are available.

Cho *et al.* (1987) developed an indirect ELISA method to detect specific IgG in cats infected with *M. yokogawai* using crude extract of metacercariae and adult worms. The results demonstrated that the serological diagnosis of metagonimiasis is feasible from the first few days of the infection. However, the sensitivity of the method was low in infections with a reduced number of worms. Lee *et al.* (1993b) used a similar method to detect metagonimiasis in humans with metacercarial crude antigens. Of the 11 metagonimiasis sera, 10 became positive to the analysis. However, cross-reactivity with other trematodiasis such as fascioliasis, schistosomiasis, and paragonimiasis was detected. A Western blot study showed also a high degree of cross-reactivity (Lee *et al.*, 1993b). Identification of antigenic specific bands was difficult. Only two bands of molecular weight

of approximately 200 kDa reacted with metagonimiasis sera and showed minimal cross-reactions (Lee *et al.*, 1993b).

Ditrich *et al.* (1991) developed indirect ELISA and Western blot methods to detect *Opisthorchis viverrini* (Opisthorchiidae) and *H. taichui* in humans using cytoplasmic and membranous antigens from adult worms of each trematode. ELISA analysis showed that cytoplasmic antigens were more sensitive, but cross-reactions between both species were found. Western blot analysis yielded several shared fractions, though differences between both trematode species were observed, enabling their differentiation. A protein band in the area of 10 kDa was characteristic of *H. taichui*.

7.5. Human Infections

Human infections by heterophyids have been often reported. Chai and Lee (2002) listed 12 species of heterophyids that parasitize humans in Korea belonging the genera *Metagonimus*, *Heterophyes*, *Stictodora*, *Heterophyopsis*, *Pygidiopsis*, *Stellantchasmus*, and *Centrocestus*. Moreover, members of the genera *Haplorchis* (Figure 13), *Phagicola*, and *Procerovum* have also been implicated in human heterophyiasis (Fried *et al.*, 2004). Chai and Lee (2002) and Fried *et al.* (2004) provide excellent coverage of human infections by these digeneans. The most prevalent species in humans are *M. yokogawai* and *H. heterophyes* (Figure 14), which are distributed mainly in Asia, Africa, and Eastern Europe (Fried *et al.*, 2004).

Humans become infected by eating raw, pickled, or poorly cooked fish. Low-grade infections are of no clinical consequence, but cases with heavy infections are associated with diarrhea, mucus-rich feces, abdominal pain, dyspepsia, anorexia, nausea, and vomiting (Marty and Andersen, 2000; Chai and Lee, 2002; Fried *et al.*, 2004). Anaphylactic reactions have also been reported (Martínez-Alonso *et al.*, 1999). Occasionally, worm eggs may enter the circulatory system though the crypts of Lieberkühn causing emboli, which may be fatal depending on the affected tissue (Marty and Andersen, 2000).

Chi *et al.* (1988) studied the pathology of a human case of metagonimiasis. The parasitism was incidentally detected in an intestinal



Figure 13 Adult worm of *Haplorchis taichui* from a human infection; the worm was stained in acetocarmine. Scale bar: 100 μ m.

segment that was removed surgically for treating intestinal perforation related to a malignant histiocytosis. The adult worms of *M. yokogawai* were found free in the jejunal lumen as well as impacted in the intervillous spaces. The pathological findings in this human case were similar to those in the recovering phase of infection in experimental animals. The main histological lesions were massive lymphoplasmacytic and eosinophilic infiltration in the stroma, erosion of the enterocytes in the areas surrounding the worms, goblet cell depletion, and occasional villous edema.



Figure 14 Adult worm of *Heterophyes heterophyes* from a human infection; the worm was stained in acetocarmine. Scale bar: 50 μ m.

8. THE FAMILY PARAMPHISTOMIDAE

8.1. Background

According to Jones (2005), the family Paramphistomidae is restricted to paramphistomoid digeneans, parasitic in mammals, which lack pharyngeal sacs, a cirrus sac and a ventral pouch. However, this family has often been used as a repository with a varying number of

subfamilies, for all paramphistomoid subfamilies and genera, from all vertebrate hosts recognized at the time (see Jones, 2005). Yamaguti (1958) referred all the paramphistomoids to the Paramphistomidae, considering 24 subfamilies. However, later studies have recognized a different number of families within Paramphistomoidea (Yamaguti, 1971; Sey, 1991; Jones, 2005). In the present section, we are concerned mainly with the members of Paramphistomidae *sensu* Jones (2005), though some data on species belonging to related families such as Gastrothylacidae or Zygocotylidae are also included.

The life cycles of most paramphistomatids involve freshwater pulmonate snails of various genera. Daughter rediae develop in the digestive gland–gonad region of the snail. Amphistome cercariae that have matured in both rediae and snail tissue emerge from the snail and encyst on a variety of aquatic vegetation. Vertebrate hosts that feed on the tainted vegetation become infected (Fried *et al.*, 2004).

8.2. Pathology of the Infection

There are numerous reports of clinical paramphistomiasis produced by a variety of species, but few describe the details of the pathogenesis of the disease (Boray, 1969; Horak, 1971). The number of worms required to produce clinical disease varies according to the species of fluke and also the species and environment of the host (Whitten, 1955; Horak and Clark, 1963; Huffman *et al.*, 1991; Rolfe *et al.*, 1994).

The gross intestinal pathology has been studied in several species of ruminants (Whitten, 1955; Boray, 1959, 1969; Varma, 1961; Buttler and Yeoman, 1962; Horak and Clark, 1963; Horak, 1967, 1971; Sharma-Deorani and Katiyar, 1967; Rolfe *et al.*, 1994) and even in rodents and domestic chicks (Fried and Nelson, 1978; Huffman *et al.*, 1991). In ruminants, the chronology of the pathological changes associated with paramphistomiasis is closely related with the development of the parasite in the definitive host. Immature paramphistomes induce pathology in the small intestine, whereas adult worms cause damage to the rumen. The severity and persistence of damage to the

small intestine depends on the number of metacercariae ingested. Horak (1971) found that the time the immature flukes spend in the small intestine is closely related to the number of flukes present. Probably, crowding delays maturation of paramphistomes and, consequently, migration to the rumen. During the first 3 WPI, the most important changes occurred in the first 3 m of the small intestine where large numbers of parasites were attached to the mucosa (Horak, 1971; Rolfe *et al.*, 1994). In sheep, the small intestine was thickened externally, hyperemic and the serosal blood vessels were prominent and congested. Internally, the intestinal rugae were prominent and often covered with a catarrhal exudate. In infections with relatively few worms, most of the worms had migrated to the rumen and were located in the abomasum or even in the anterior ruminal pillar (Rolfe *et al.*, 1994). At 6 WPI, the mucosa was hyperemic, thickened, and with ecchymotic hemorrhages up to 6 m from the pylorus, although most of the worms were located in the proximal region of the intestine (Rolfe *et al.*, 1994). At 12 WPI, the wall of the small intestine was thickened, the mucosa was pale and with prominent rugae (Rolfe *et al.*, 1994).

Intestinal histopathology of paramphistomiasis has been studied in sheep, goat, and cattle (Boray, 1959, 1969; Horak, 1967, 1971; Sharma-Deorani and Katiyar, 1967; Singh and Lakra, 1971; Singh *et al.*, 1984; Sahai *et al.*, 1985; Rolfe *et al.*, 1994). Histopathological changes were found in the first 8 m of the small intestine, though the pathology was more severe 2-4 m from the pylorus. The villi were shortened and thickened and even were destroyed in some areas in the late phases of intestinal paramphistomiasis. The area occupied by submucosal glands was increased and cystic dilation of Brunner's glands also occurred (Singh *et al.*, 1984; Rolfe *et al.*, 1994). There was an increased cellularity in the lamina propria and in the intestinal tissue occupied by Brunner's glands. Significant infiltration of macrophages, lymphocytes, and fibroblasts was observed. In sheep infected with *Paramphistomum ichikawai*, a large number of eosinophils was present at most levels of the intestine, despite the absence on worms in these sites. Mast cells were depleted in areas where the flukes were present. An increased number of neutrophils in association with tissue destruction was also observed. At 6-12 WPI, the

number of plasma cells was increased and goblet cells were expelling mucin (Rolfe *et al.*, 1994). At 12 WPI, the worms had migrated to the rumen and most of the small intestinal mucosal changes were resolved. However, some areas of the intestinal mucosa were thickened and contained fibroblasts and macrophages. Leukocytes and mast cells were also abundant 3–4 m distal from the pylorus (Rolfe *et al.*, 1994).

The importance of changes caused by adult paramphistomes in the rumen is unclear. Most authors suggest that worms in the rumen do not cause significant clinical or subclinical effects (Horak, 1971; Singh *et al.*, 1984). However, Rolfe *et al.* (1994) showed that ruminal lesions caused by *P. ichikawai* in sheep may be important. At 12 WPI, the rumen was affected adjacent to the dorsal ruminal pillar. Papillae were fused into aggregations and the stratum corneum and granulosa were thinner at the site of worm attachment. Eosinophils were abundant in the lamina propria adjacent to the areas of attachment. No changes were seen in the abomasum. Singh *et al.* (1984) also described an infiltration of mononuclear cells in the connective tissue of the rumen of goats experimentally infected with *P. cervi*.

8.3. Immunology of the Infection

8.3.1. Manifestations of Resistance to Infection

The findings of several authors support the idea that infection with paramphistomes provides a certain degree of protection against reinfection and its clinical effects (Whitten, 1955; Boray, 1959, 1969; Horak, 1967, 1971). Boray (1959) showed that paramphistomiasis rarely occurs in adult cattle, probably in relation to an earlier infection which provided a certain degree of immunity. Boray (1969) found that older sheep had fewer adult worms of *P. ichikawai* than younger ones and were not affected by the disease during an outbreak of paramphistomiasis in Australia. In cattle, Whitten (1955) and Horak (1967) noted that previous infection may induce a degree of resistance capable of withstanding the massive infection required to produce paramphistomiasis in the wild. In the laboratory,

successful immunization against massive experimental infection with *P. microbothrium* has been reported (Horak, 1971). This immunity also protected the host from the clinical effects of paramphistomiasis. Moreover, the growth rate of worms in challenge infections was retarded and the migration from the small intestine to the rumen was delayed (Horak, 1967, 1971).

The development of an effective immunity against paramphistome infections is dependent upon a number of factors (Horak, 1967, 1971). Immunity depends upon the metacercarial dose used as an immunizing infection but not upon the number of worms present in the rumen as demonstrated by immunization with X-irradiated metacercariae. The X-irradiated metacercariae were capable of excystation and attachment but many juvenile worms were lost during and after worm migration (Horak, 1971; Hafeez and Rao, 1981, 1983). Cattle immunized with X-irradiated metacercariae of *P. microbothrium* were practically immune to reinfection. Moreover, the immunity also depended upon the presence of worms. If the worms resulting from the immunizing infection were removed by anthelmintic treatment, the degree of immunity was reduced considerably (Horak, 1967, 1971).

8.3.2. Effector Mechanisms of the Immune Response

Little is known about the effector mechanisms involved in the immune response against paramphistomes. The number of different cell types has been found to be increased in association with paramphistome infection (Horak, 1971; Rolfe *et al.*, 1994). An increase in the number of eosinophils was found in sheep experimentally infected with *P. ichikawai* coinciding with the time at which most worms were rejected (Rolfe *et al.*, 1994). However, the role of these eosinophils in the expulsion of the worms was unclear. The number of globular leukocytes also increased in infected sheep relative to the non-infected controls. This cell type appeared throughout the small intestine but only after the worms had migrated to the rumen, suggesting that an initial sensitization was required before globular leukocytes were recruited. Rolfe *et al.* (1994) suggested that globular leukocytes may be

involved in a delayed inflammatory response and parasite rejection. The number of plasma cells were also increased in paramphistome infections (Boch *et al.*, 1983; Rolfe *et al.*, 1994). However, Boch *et al.* (1983) found that the role of secretory antibodies from plasma cells in the expulsion of *P. cervi* was uncertain. Horak (1967) found precipitating antibodies in sera from sheep, goat, and cattle infected with *P. microbothrium*. The antibody levels were particularly high in cattle harboring adult worms and were lower only when immature flukes were present. Cattle developed complete resistance to reinfection, though the role of humoral immunity was not well understood since some rejection occurred even before the appearance of mucosal plasma cells and circulating antibodies.

8.3.3. Antigenic Characterization

Several studies have been done to analyze the polypeptide composition of paramphistome species. These studies have shown a close antigenic relationship between the species within the Paramphistomidae. Cross-reactivity between different species of paramphistomes has been demonstrated by agar-gel precipitation, Ouchterlony double immunodiffusion, and immunoelectrophoresis (Varma *et al.*, 1991; Maji *et al.*, 1997a, b, 1998, 1999). Furthermore, cross-reactivity with *Fasciola* spp. and *Schistosoma mattheei* has also been reported (Horak, 1967; Goubadia and Fagbemi, 1996; Viyanant *et al.*, 1997; Ibarra *et al.*, 1998; Yadav *et al.*, 2003).

Saifullah *et al.* (2000a) analyzed the somatic and excretory-secretory products of adult worms of *Gastrothylax crumenifer* collected from Indian water buffaloes. The results of gradient SDS-PAGE profiles revealed a total of 41 and 38 major polypeptides, weighing from 29 to 205 kDa, in somatic and excretory-secretory products, respectively. A total of 14 polypeptides was common to both types of antigens. Only a small number of polypeptides reacted with hyperimmune rabbit antisera raised against somatic and excretory-secretory antigens based on immunoblotting analysis. Saifullah *et al.* (2000b) partially purified and characterized the somatic antigens of *G. crumenifer*. Eight major fractions were separated on a Sephadex column from adult worm

whole homogenate. The analysis of individual fractions by SDS-PAGE and Western blot using hyperimmune rabbit antisera, showed that the most immunogenic fractions were those of low molecular weight in the range of 14–40 kDa.

8.4. Immunodiagnosis

Several works have attempted to develop tools for the immunodiagnosis of paramphistomiasis in ruminants. However, these methods lack specificity and are difficult to interpret (Horak, 1967, 1971; Kumari and Hafeez, 2004). Horak (1967) showed that immunodiagnosis of *P. microbothrium* infections is feasible using adult worms. Incubation of adult and immature worms with serum of infected sheep, goat, or cattle gave precipitates at the excretory pores, genital atrium, and on the anterior worm tegument. This method, however, did not differentiate between an infected animal requiring treatment or an animal immune to reinfection (Horak, 1967, 1971).

Horak (1967) also developed an intradermal test to diagnose *P. microbothrium* in sheep using three different antigens: saline extract of immature worms, saline extract of metacercariae, and alcohol-precipitated antigen of adult and immature worms. A positive reaction showed the appearance of a dark purple area at the site of the injection surrounded by an edematous weal. Although the saline extracts gave the most reliable results, none of the three antigens was specific since positive reactions were also observed in some sheep infected with *F. hepatica* or *S. mattheei*.

Horak (1967) used a modified complement fixation test to detect *P. microbothrium* in sheep. He used boiled alcohol-precipitated extracts from adult and immature worms. Positive results were obtained in infected sheep. This method, however, is not practical for routine diagnosis because of difficulties in interpreting the results.

8.5. Human Infections

Within the paramphistomes (including Gastrodiscidae), only three genera have been reported to parasitize humans (see Table 1).

Gastrodiscoides hominis infects people and their livestock (e.g., hogs) in Africa and South-East Asia, mainly in India, *Watsonius watsoni* in Africa, and *Fischoederius elongates* in China (Boray, 1982; Marty and Andersen, 2000; Fried *et al.*, 2004). It is suspected that humans become infected by eating aquatic vegetation and crustaceans and amphibians. Marty and Andersen (2000) note that heavy infection with paramphistomes can produce headache, epigastric pain, and diarrhea that may be a reaction to metabolites released by the flukes, but the immunology and pathology of these infection in humans have not been studied.

9. OTHER FAMILIES OF INTEREST

In the previous sections, we have reviewed salient information on the immunological and pathological effects of the families of intestinal trematodes for which there is a sufficient body of published work. However, there are other families of intestinal trematodes that have not received much attention or for which only scarce information has been published in relation to these topics. The present section provides some information on intestinal trematodes not discussed in the body of the work because of the limited published information on these groups. In fact, most of these studies discussed herein are limited to reports of intestinal trematodes infecting different hosts in the wild.

There are numerous reasons that help explain the lack of research on the immunology and pathology of intestinal trematodes. Most of these trematode species are not considered of clinical or economic importance because they infect only wild animals; moreover, many of the infections are only associated with minor symptoms. Furthermore, the human infections are often locally endemic and their epidemiological importance is not well recognized. For instance, *Fasciolopsis buski* (Fasciolidae) is one of the most evident examples. Although, *F. buski* has been reported frequently in humans, mainly in the Far East and the Southeast Asia (Graczyk *et al.*, 2001; Fried *et al.*, 2004), only scarce data are available on the pathological and immunological effects of these infections. Clinical symptoms in

F. buski infections in humans are related to parasite load and, in light infections, may include anemia, eosinophilia, dizziness, and gastrointestinal symptoms (Gilman *et al.*, 1982; Fried *et al.*, 2004). Adult flukes damage the intestinal mucosa and cause extensive duodenal erosions, ulceration, hemorrhage, abscesses, and catarrhal inflammation. Absorption of toxic and allergic worm metabolites causes ascitis, general edema, and facial edema (Jaroovvesama *et al.*, 1986; Graczyk *et al.*, 2001; Fried *et al.*, 2004).

There are also some data available on the pathology caused by intestinal trematodes in waterfowl, particularly in relation to death of birds in the wild. Most of these data were obtained from observations in the wild and only a few laboratory studies have been made. *Sphaeridotrema globulus* (Psilostomidae) causes fatal enteritis in birds (Roscoe and Huffman, 1982, 1983; Huffman *et al.*, 1984). This species parasitizes the duodenum and jejunum of waterfowl such as the cygnet swan (*Cygnus olor*). At the sites of infection, adult worms ulcerate the intestine and severe hemorrhage from damaged sub-mucosal capillaries was observed. A mononuclear periportal hepatitis and secondary nodules in the spleen were commonly observed. Infected swans died from hypovolemic shock or other complications associated with the infection (Roscoe and Huffman, 1982; Huffman *et al.*, 1984). Increases in mast cells and eosinophils have been reported in ducks (*Anas platyrrhynchos*) experimentally infected with *S. globulus* (Mucha and Huffman, 1991). *Cyathocotyle bushiensis* (Cyathocotylidae) is another intestinal trematode frequently reported in ducks and has been associated with the death of these birds (Gagnon *et al.*, 1993). Infection was often associated with decreased weight gain and a minor increase in body temperature. Moreover, increased permeability of the cecal wall to vascular products, elevation in hemoglobin concentrations, and packed cell volume were also observed in infected ducks (Gagnon *et al.*, 1993).

10. CONCLUDING REMARKS

This review introduces the reader to the pathological and immunological aspects of intestinal trematodes in their definitive hosts. Many

of the species covered in this review are of medical and/or veterinary importance and may cause disease in humans and animals. Information on the topics covered in the review is relatively sparse compared with that on other helminths, particularly the intestinal nematodes.

The review considers work on different species of intestinal trematodes with an emphasis on experimental studies that may provide useful information on the factors that determine the pathology caused by these parasites during the course of infection. The pathology and immunology of intestinal trematodiasis has been poorly studied. It is difficult to ascertain the reasons behind the lack of research on these topics. Many of the species are not considered of clinical importance and are only locally endemic. This may explain in part the minimal attention given to these helminths. However, many of the species considered in the review cause significant pathology and elicit an immune response. In some cases the immune response causes an early worm rejection, whereas in other cases chronic infections are established; for most situations, a relationship between the immune effector mechanism and the course of the infection has not been determined. Under laboratory conditions, studies have indicated that the pathology caused by intestinal trematodes and the course of the infection depends on several factors related to both the species of host and parasite. Further studies are required to understand the mechanisms involved in these responses. Although several aspects, e.g., cytokine profiles, remain to be studied, many of the species considered in this review offer the possibility of comparative studies on the development of a single trematode species in different host species. Such studies, using intestinal trematodes as experimental models, may be useful to analyze the immune mechanisms involved in parasite rejection.

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REFERENCES

- Abe, T., Sugaya, H. and Yoshimura, K. (1993). Different susceptibility to the IL-3 induced-protective effects between *Strongyloides ratti* and *Nippostrongylus brasiliensis* in C57BL/6 mice. *Parasite Immunology* **15**, 643–645.
- Abou-Zakham, A.A., Makhoul, L., el-Kholi, E.I., Abo-Shady, A.F., Abdel-Magied, S.A. and Youssef, M.E. (1987). Sero immunological diagnosis of heterophyiasis. *Journal of the Egyptian Society of Parasitology* **17**, 569–572.
- Agger, M.K., Simonsen, P.E. and Vennervald, B.J. (1993). The antibody response in serum, intestinal wall and intestinal lumen of NMRI mice infected with *Echinostoma caproni*. *Journal of Helminthology* **67**, 169–178.
- Ahn, H., Rim, H.J. and Kim, S.J. (1991). [Antigenic localities in the tissues of *Metagonimus yokogawai* observed by immunogold labeling method] (in Korean). *The Korean Journal of Parasitology* **29**, 245–257.
- Andresen, K., Simonsen, P.E., Andersen, B.J. and Birch-Andersen, A. (1989). *Echinostoma caproni* in mice, shedding of antigens from the surface of an intestinal trematode. *International Journal for Parasitology* **19**, 111–118.
- Auriat, C., Ouassii, M.A., Torpier, G., Eisen, H. and Capron, A. (1981). Proteolytic cleavage of IgG bound to Fc receptor of *Schistosoma mansoni* schistosomula. *Parasite Immunology* **3**, 33–44.
- Avila, G., Benitez, M., Aguilar-Vega, L. and Flisser, A. (2003). Kinetics of *Taenia solium* antibodies and antigens in experimental taeniosis. *Parasitology Research* **89**, 284–289.
- Balic, A., Bowles, V.M. and Meeusen, E.N. (2000). The immunobiology of gastrointestinal nematode infections in ruminants. *Advances in Parasitology* **45**, 181–241.
- Bandyopadhyay, A.K., Maji, A.K., Manna, B., Bera, D.K., Addy, M. and Nandy, A. (1995). Pathogenicity of *Artyfechinostomum oraoni* in naturally infected pigs. *Tropical Medicine and Parasitology* **46**, 138–139.
- Beaver, P.C., Little, M.D., Tucker, C.F. and Reed, R.J. (1977). Mesocercaria in the skin of man in Louisiana. *American Journal of Tropical Medicine and Hygiene* **26**, 422–426.

- Berasain, P., Carmona, C., Frangione, B., Dalton, J.P. and Goni, F. (2000). *Fasciola hepatica*: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. *Experimental Parasitology* **94**, 99–110.
- Bindseil, E. and Christensen, N.Ø. (1984). Thymus-independent crypt hyperplasia and villous atrophy in the small intestine of mice infected with the trematode *Echinostoma revolutum*. *Parasitology* **88**, 431–438.
- Boch, J., Schmid, K., Ruckrich, H.U., Erich, E., Keller, B., Weiland, G. and Gobel, E. (1983). Stomach fluke (*Paramphistomum cervi*) infection of ruminants. *Berliner und Münchener Tierärztliche Wochenschrift* **96**, 338–346.
- Boray, J.C. (1959). Studies on intestinal amphistomiasis in cattle. *Australian Veterinary Journal* **35**, 282–287.
- Boray, J.C. (1969). Studies on intestinal paramphistomosis in sheep due to *Paramphistomum ichikawai*. Fukui, 1922. *Veterinary Medical Review* **4**, 290–308.
- Boray, J.C. (1982). Paramphistomiasis. In: *Handbook Series in Zoonosis, Section C. Parasitic Zoonoses* (G.V. Hillyer and C.E. Hopla, eds). Vol. 3. pp. 167–175. Boca Raton: CRC Press.
- Brunet, L.R., Joseph, S., Dunne, D.W. and Fried, B. (2000). Immune responses during the acute stages of infection with the intestinal trematode *Echinostoma caproni*. *Parasitology* **120**, 565–571.
- Butcher, A.R. and Grove, D.I. (2001). Description of the life-cycle stages of *Brachylaima cribbi* n. sp. (Digenea: Brachylaimidae) derived from eggs recovered from human faeces in Australia. *Systematic Parasitology* **49**, 211–221.
- Butcher, A.R., Palethorpe, H.M. and Grove, D.I. (2002a). The susceptibility of inbred mice to infection with *Brachylaima cribbi* (Digenea: Brachylaimidae). *Parasitology International* **51**, 109–115.
- Butcher, A.R., Palethorpe, H.M. and Grove, D.I. (2002b). Effects of sex and age on the susceptibility of C57BL 6J mice to infection with *Brachylaima cribbi* and the course of the infection in NOD SCID mice. *Parasitology Research* **88**, 668–674.
- Butcher, A.R., Palethorpe, H.M. and Grove, D.I. (2003). Response to re-infection with *Brachylaima cribbi* in immunocompetent and immunodeficient mice. *Parasitology International* **52**, 219–228.
- Butcher, A.R., Parasuramar, P.S., Thompson, C.S. and Grove, D.I. (1998). First report of the isolation of an adult worm of the genus *Brachylaima* (Digenea: Brachylaimidae), from the gastrointestinal tract of a human. *International Journal for Parasitology* **28**, 607–610.
- Butcher, A.R., Talbot, G.A., Norton, R.E., Kirk, M.D., Cribb, T.H., Forsyth, J.R.L., Knight, B. and Cameron, A.S. (1996). Locally acquired *Brachylaima* sp. (Digenea: Brachylaimidae) intestinal fluke infection

- in two South Australian infants. *Medical Journal of Australia* **164**, 475–478.
- Buttler, R.W. and Yeoman, G.H. (1962). Acute intestinal paramphistomiasis in zebu cattle in Tanganyika. *Veterinary Record* **74**, 227–231.
- Chai, J.Y. (1979). Study on *Metagonimus yokogawai* (Katsurada, 1912) in Korea. V. Intestinal pathology in experimentally infected albino rats. *Seoul Journal of Medicine* **20**, 104–117.
- Chai, J.Y., Choi, M.H., Yu, J.R. and Lee, S.H. (2003). *Gymnophalloides seoi*, a new human intestinal trematode. *Trends in Parasitology* **19**, 109–112.
- Chai, J.Y., Chung, W.J., Kook, J., Seo, M., Park, Y.K., Guk, S.M., Choi, M.H. and Lee, S.H. (1999). Growth and development of *Gymnophalloides seoi* in immunocompetent and immunosuppressed C3H HeN mice. *The Korean Journal of Parasitology* **37**, 21–26.
- Chai, J.Y., Hong, S.T., Lee, S.H., Lee, G.C. and Min, Y.I. (1994a). A case of echinostomiasis with ulcerative lesions in the duodenum. *The Korean Journal of Parasitology* **32**, 201–204.
- Chai, J.Y., Kim, T.K., Cho, W.H., Seo, M., Kook, J., Guk, S.M. and Lee, S.H. (1998). Intestinal mastocytosis and goblet cell hyperplasia in BALB/c and C3H mice infected with *Neodiplostomum seoulense*. *The Korean Journal of Parasitology* **36**, 109–119.
- Chai, J.Y., Kim, T.H., Kho, W.G., Chung, S.W., Hong, S.T. and Lee, S.H. (1993). Mucosal mast cell responses to experimental *Metagonimus yokogawai* infection in rats. *The Korean Journal of Parasitology* **31**, 129–134.
- Chai, J.Y., Kim, J. and Lee, S.H. (1995). Invasion of *Metagonimus yokogawai* into the submucosal layer of the small intestine of immunosuppressed mice. *The Korean Journal of Parasitology* **33**, 313–321.
- Chai, J.Y. and Lee, S.H. (1991). Intestinal trematodes infecting humans in Korea. *Southeast Asian Journal of Tropical Medicine and Public Health* **22**, 163–170.
- Chai, J.Y. and Lee, S.H. (2002). Food-borne intestinal trematode infections in the Republic of Korea. *Parasitology International* **51**, 129–154.
- Chai, J.Y., Lee, H.S., Hong, S.J., Yoo, J.H., Guk, S.M., Seo, M., Choi, M.H. and Lee, S.H. (2001). Intestinal histopathology and in situ postures of *Gymnophalloides seoi* in experimentally infected mice. *The Korean Journal of Parasitology* **39**, 31–41.
- Chai, J.Y., Lee, G.C., Park, Y.K., Han, E.T., Seo, M., Kim, J., Guk, S.M., Shin, E.H., Choi, M.H. and Lee, S.H. (2000b). Persistent endemicity of *Gymnophalloides seoi* infection in a southwestern coastal village of Korea with special reference to its egg laying capacity in the human host. *The Korean Journal of Parasitology* **38**, 51–57.
- Chai, J.Y., Seo, B.S. and Lee, S.H. (1984). Study on *Metagonimus yokogawai* (Katsurada, 1912) In Korea. VII. Susceptibility of various

- strains of mice to *Metagonimus* infection and effect of prednisolone. *The Korean Journal of Parasitology* **22**, 153–160.
- Chai, J.Y., Shin, E.H., Han, E.T., Guk, S.M., Choi, M.H. and Lee, S.H. (2000a). Genetic difference in susceptibility and fatality of three strains of mice experimentally infected with *Neodiplostomum seoulense*. *Journal of Parasitology* **86**, 1140–1144.
- Chai, J.Y., Yun, T.Y., Kim, J., Huh, S., Choi, M.H. and Lee, S.H. (1994b). [Chronological observation on intestinal histopathology and intraepithelial lymphocytes in the intestine of rats infected with *Metagonimus yokogawai*] (in Korean). *The Korean Journal of Parasitology* **32**, 215–221.
- Chattopadhyay, U.K., Das, M.S., Pal, D., Das, S. and Mukherjee, A. (1990). A case of echinostomiasis in tribal community in Bengal. *Annales of Tropical Medicine and Parasitology* **84**, 193.
- Chi, J.G., Kim, C.W., Kim, J.R. and Hong, S.T. (1988). Intestinal pathology in human metagonimiasis with ultrastructural observations of parasites. *Journal of Korean Medical Science* **3**, 171–177.
- Ching, H.L. (1995). Evaluation of characters of the digenean family Gymnophallidae Morozov, 1955. *Canadian Journal of Fisheries and Aquatic Science* **52**, 78–83.
- Cho, S.Y., Kim, S.I., Earm, Y.E. and Ho, W.K. (1985). A preliminary observation on watery content of small intestine in *Metagonimus yokogawai* infected dog. *The Korean Journal of Parasitology* **23**, 175–177.
- Cho, S.Y., Kim, S.I. and Kang, S.Y. (1987). Specific IgG responses in experimental cat metagonimiasis. *The Korean Journal of Parasitology* **25**, 149–153.
- Cho, Ch.M., Tak, W.Y., Kweon, Y.O., Kim, S.K., Choi, Y.H., Kong, H.H. and Chung, D.I. (2003). A human case of *Echinostoma hortense* (Trematoda: Echinostomatidae) infection diagnosed by gastroduodenal endoscopy in Korea. *The Korean Journal of Parasitology* **41**, 117–120.
- Choi, M.H., Chai, J.Y. and Lee, S.H. (1998a). Purification and characterization of a 16-kDa cysteine proteinase of *Gymnophalloides seoi* (Gymnophallidae) metacercariae. *Journal of Parasitology* **84**, 350–353.
- Choi, M.H., Choe, S.C. and Lee, S.H. (1999). A 54 kDa cysteine protease purified from the crude extract of *Neodiplostomum seoulense* adult worms. *The Korean Journal of Parasitology* **37**, 39–46.
- Choi, M.H., Park, W.J., Park, Y.K., Chai, J.Y. and Lee, S.H. (1998b). Isolation and characterization of a 40 kDa cysteine protease from *Gymnophalloides seoi* adult worms. *Korean Journal of Parasitology* **36**, 133–141.
- Christensen, N.Ø., Fagbemi, B.O. and Nansen, P. (1984). Trypanosoma brucei-induced blockage of expulsion of *Echinostoma revolutum* and of homologous *E. revolutum* resistance in mice. *Journal of Parasitology* **70**, 558–561.
- Christensen, N.Ø., Knudsen, J., Fagbemi, B.O. and Nansen, P. (1985). Impairment of primary expulsion of *Echinostoma revolutum* in mice

- concurrently infected with *Schistosoma mansoni*. *Journal of Helminthology* **59**, 333–335.
- Chung, P.R., Jung, Y. and Kim, D.S. (1996). *Segmentina* (*Polypylis*) *hemisphaerula* (Gastropoda: Planorbidae): a new molluscan intermediate host of a human intestinal fluke *Neodiplostomum seoulensis* (Trematoda: Diplostomatidae) in Korea. *Journal of Parasitology* **82**, 336–338.
- Cribb, T.H., Bray, R.A., Olson, P.D. and Littlewood, D.T. (2003). Life cycle evolution in the digenea: a new perspective from phylogeny. *Advances in Parasitology* **54**, 197–254.
- Ditrich, O., Kopacek, P., Giboda, M., Gutvirth, J. and Scholz, T. (1991). Serological differentiation of human small fluke infections using *Opisthorchis viverrini* and *Haplorchis taichui* antigens. *Southeast Asian Journal of Tropical Medicine and Public Health* **22**, 174–178.
- Dubois, G. (1970). Synopsis des Strigeidae et Diplostomatidae (Trematoda). *Mémoires de la Société Neuchâteloise des Sciences Naturelles* **10**, 259–727.
- El-Ganayni, G.A., Youssef, M.E., Handousa, A.E., Abou-Zakham, A.A. and Hegazi, M.M. (1989). Serum and intestinal immunoglobulins in heterophyiasis. *Journal of the Egyptian Society of Parasitology* **19**, 219–223.
- El-Ridi, A.M., Hegazi, M.M., Atia, M.M., El-Gamal, R.L. and Nada, S.M. (1990). Cell-mediated immune response in experimental infection with *H. heterophyes*. *Journal of the Egyptian Society of Parasitology* **20**, 243–249.
- Fernandes, B.J., Cooper, J.D., Cullen, J.B., Freeman, R.S., Ritchie, A.C., Scott, A.A. and Stuart, P.F. (1976). Systemic infection with *Alaria americana* (Trematoda). *Canadian Medical Association Journal* **115**, 1111–1114.
- Finkelman, F.D., Shea-Donohue, T., Goldhill, J., Sullivan, C.A., Morris, S.C., Madden, K.B., Gause, W.C. and Urban, J.F., Jr. (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annual Reviews in Immunology* **15**, 505–533.
- Franco, J., Huffman, J.E. and Fried, B. (1986). Infectivity, growth, and development of *Echinostoma revolutum* (Digenea: Echinostomatidae) in the golden hamster, *Mesocricetus auratus*. *Journal of Parasitology* **72**, 142–147.
- Frazer, B.A., Fried, B., Fujino, T. and Sleckman, B.P. (1999). Host-parasite relationships between *Echinostoma caproni* and RAG-2-deficient mice. *Parasitology Research* **85**, 337–342.
- Freeman, R.S., Stuart, P.F., Cullen, J.B., Ritchie, A.C., Mildon, A., Fernandes, B.J. and Bonin, R. (1976). Fatal human infection with mesocercariae of the trematode *Alaria americana*. *American Journal of Tropical Medicine and Hygiene* **25**, 803–807.
- Fried, B. and Graczyk, T.K. (2004). Recent advances in the biology of *Echinostoma* species in the “*revolutum*” group. *Advances in Parasitology* **58**, 139–195.

- Fried, B., Graczyk, T.K. and Tamang, L. (2004). Food-borne intestinal trematodiasis in humans. *Parasitology Research* **93**, 159–170.
- Fried, B. and Huffman, J.E. (1996). The biology of the intestinal trematode *Echinostoma caproni*. *Advances in Parasitology* **38**, 311–368.
- Fried, B., Huffman, J.E. and Weiss, P.M. (1990). Single and multiple worm infections of *Echinostoma caproni* (Trematoda) in the golden hamster. *Journal of Helminthology* **64**, 75–78.
- Fried, B. and Nelson, P.D. (1978). Host-parasite relationships of *Zygocotyle lunata* (Trematoda) in the domestic chick. *Parasitology* **77**, 49–55.
- Fujino, T. and Fried, B. (1993a). Expulsion of *Echinostoma trivolvis* (Cort, 1914) Kanev, 1985 and retention of *E. caproni* Richard, 1964 (Trematoda, Echinostomatidae) in C3H mice: pathological, ultrastructural, and cytochemical effects on the host intestine. *Parasitology Research* **79**, 286–292.
- Fujino, T. and Fried, B. (1993b). *Echinostoma caproni* and *E. trivolvis* alter the binding of glycoconjugates in the intestinal mucosa of C3H mice as determined by lectin histochemistry. *Journal of Helminthology* **67**, 179–188.
- Fujino, T. and Fried, B. (1996). The expulsion of *Echinostoma trivolvis* from C3H mice: differences in glycoconjugates in mouse versus hamster small intestinal mucosa during infection. *Journal of Helminthology* **70**, 115–121.
- Fujino, T., Fried, B. and Hosier, D.W. (1994). The expulsion of *Echinostoma trivolvis* (Trematoda) from ICR mice: extension retraction mechanisms and ultrastructure of the collar spines. *Parasitology Research* **80**, 581–587.
- Fujino, T., Fried, B., Ichikawa, H. and Tada, I. (1996a). Rapid expulsion of the intestinal trematodes *Echinostoma trivolvis* and *E. caproni* from C3H mice by trapping with increased goblet cell mucins. *International Journal for Parasitology* **26**, 319–324.
- Fujino, T., Fried, B. and Tada, I. (1993). The expulsion of *Echinostoma trivolvis*: worm kinetics and intestinal cytopathology in conventional and congenitally athymic BALB c mice. *Parasitology* **106**, 297–304.
- Fujino, T., Ichikawa, H. and Fried, B. (1998a). The immunosuppressive compound FK506 does not affect expulsion of *Echinostoma trivolvis* in C3H mice. *Parasitology Research* **84**, 519–521.
- Fujino, T., Ichikawa, H., Fried, B. and Fukuda, K. (1996b). The expulsion of *Echinostoma trivolvis*: suppressive effects of dexamethasone on goblet cell hyperplasia and worm rejection in C3H/HeN mice. *Parasite* **3**, 283–289.
- Fujino, T., Ichikawa, H., Fried, B. and Fukuda, K. (1997). The expulsion of *Echinostoma trivolvis*: worm kinetics and intestinal reactions in C3H HeN mice treated with dexamethasone. *Journal of Helminthology* **71**, 257–259.
- Fujino, T., Ichikawa, H., Fukuda, K. and Fried, B. (1998b). The expulsion of *Echinostoma trivolvis* caused by goblet cell hyperplasia in severe combined immunodeficient (SCID) mice. *Parasite* **5**, 219–222.

- Fujino, T., Yamada, M., Ichikawa, H., Fried, B., Arizono, N. and Tada, I. (1996c). Rapid expulsion of the intestinal trematodes *Echinostoma trivolvis* and *E. caproni* from C3H HeN mice after infection with *Nippostrongylus brasiliensis*. *Parasitology Research* **82**, 577–579.
- Gagnon, C., Scott, M.E. and McLaughlin, J.D. (1993). Gross lesions and hemathological changes in domesticated mallard ducklings experimentally infected with *Cyathocotyle bushiensis* (Digenea). *Journal of Parasitology* **79**, 757–762.
- Garside, P., Kennedy, M.W., Wakelin, D. and Lawrence, C.E. (2000). Immunopathology of intestinal helminth infection. *Parasite Immunology* **22**, 605–612.
- Gibson, D.I. (1996). Trematoda. In: *Guide to the Parasites of Fishes of Canada, Part IV, Canadian Special Publication of Fisheries and Aquatic Sciences* (L. Margolis and Z. Kabata, eds), No. 124, pp. 1–373. Ottawa: NRC Press.
- Gibson, D.I. and Bray, R.A. (1994). The evolutionary expansion and host-parasite relationship of the Digenea. *International Journal for Parasitology* **24**, 1213–1226.
- Gilman, R.H., Mondal, G., Maksud, M., Alan, K., Rutherford, E., Gilman, J.B. and Khan, M.U. (1982). Endemic focus of *Fasciolopsis buski* infection in Bangladesh. *American Journal of Tropical Medicine and Hygiene* **31**, 796–802.
- Graczyk, T.K. and Fried, B. (1994). ELISA method for detecting anti-*Echinostoma caproni* (Trematoda: Echinostomatidae) immunoglobulins in experimentally infected ICR mice. *Journal of Parasitology* **80**, 544–549.
- Graczyk, T.K. and Fried, B. (1995). An enzyme-linked immunosorbent assay for detecting anti-*Echinostoma trivolvis* (Trematoda) IgG in experimentally infected ICR mice. Cross-reactivity with *E. caproni*. *Parasitology Research* **81**, 710–712.
- Graczyk, T.K. and Fried, B. (1998). Echinostomiasis: a common but forgotten food-borne disease. *American Journal of Tropical Medicine and Hygiene* **58**, 501–504.
- Graczyk, T., Gilman, R.H. and Fried, B. (2001). Fasciolopsiasis: is it a controllable food-borne disease? *Parasitology Research* **87**, 80–83.
- Griffiths, H.J., Gonder, E. and Pomeroy, B.S. (1976). An outbreak of trematodiasis in domestic geese. *Avian Disease* **20**, 604–606.
- Guk, S.M., Park, J.Y., Seo, M., Han, E.T., Kim, J.L. and Chai, J.Y. (2005). Susceptibility of inbred mouse strains to infection with three species of *Metagonimus* prevalent in the Republic of Korea. *Journal of Parasitology* **91**, 12–16.
- Guobadia, E.E. and Fagbemi, B.O. (1996). Detection of circulating *Fasciola gigantica* antigen in experimental and natural infections of sheep with fasciolosis. *Veterinary Parasitology* **65**, 29–39.

- Haaseb, M.A. and Eveland, L.K. (2000). Human echinostomiasis: mechanisms of pathogenesis and host resistance. In: *Echinostomes as Experimental Models for Biological Research* (B. Fried and T.K. Graczyk, eds), pp. 83–98. Dordrecht: Kluwer Academic Publishers.
- Hafeez, M. and Rao, B.V. (1981). Studies on amphistomiasis in Andhra Pradesh (India) VI. Immunization of lambs and kids with gamma irradiated metacercariae of *Cercariae indicae* XXVI. *Journal of Helminthology* **55**, 29–32.
- Hafeez, M. and Rao, B.V. (1983). Immunological studies of amphistomiasis in sheep and goats. II. Viability of amphistome metacercariae after irradiation. *Journal of Helminthology* **57**, 115–116.
- Hansen, K., Nielsen, J.W., Hindsbo, O. and Christensen, N.Ø. (1991). *Echinostoma caproni* in rats: worm population dynamics and host blood eosinophilia during primary infections with 6, 25 and 50 metacercariae and resistance to secondary and superimposed infections. *Parasitology Research* **77**, 686–690.
- Hassan, M.M., Farghaly, A.M., El-Gamal, R.L. and El-Ridi, A.M. (1989). Cross-reactions in immunodiagnosis of patients infected with *Schistosoma*, *Fasciola* and *Heterophyes* using ELISA. *Journal of the Egyptian Society of Parasitology* **19**, 845–851.
- Hayes, K.S., Bancroft, A.J. and Grencis, R.K. (2004). Immune-mediated regulation of chronic intestinal nematode infection. *Immunological Reviews* **201**, 75–88.
- Hong, S.T. (1982). Studies on intestinal trematodes in Korea VII. Growth, development and recovery of *Fibricola seoulensis* from experimentally infected rats and mice. *The Korean Journal of Parasitology* **20**, 112–121.
- Hong, S.T., Chai, J.Y. and Lee, S.H. (1984a). Ten human cases of *Fibricola seoulensis* infection and mixed one with *Stellantchasmus* and *Metagonimus*. *The Korean Journal of Parasitology* **24**, 95–97.
- Hong, S.T., Cho, T.K., Hong, S.J., Chai, J.Y., Lee, S.H. and Seo, B.S. (1984b). Fifteen human cases of *Fibricola seoulensis* infection in Korea. *The Korean Journal of Parasitology* **22**, 61–65.
- Hong, S.J., Han, J.H., Park, C.K. and Kang, S.Y. (1997). Intestinal pathologic findings at early stage infection by *Centrocestus armatus* in albino rats. *The Korean Journal of Parasitology* **35**, 135–138.
- Hong, S.T., Hong, S.J., Lee, S.H., Seo, B.S. and Chi, J.G. (1982). Studies on intestinal trematodes in Korea: VI. On the metacercaria and the second intermediate host of *Fibricola seoulensis*. *The Korean Journal of Parasitology* **20**, 101–111.
- Hong, S.J., Lee, S.H., Seo, B.S., Hong, S.T. and Chai, J.Y. (1983). Studies on intestinal trematodes in Korea: IX. Recovery rate and development of *Fibricola seoulensis* in experimental animals. *The Korean Journal of Parasitology* **21**, 224–233.

- Hong, S.T. and Shoop, W.L. (1995). *Neodiplostomum seoulense*, the emended name for *Neodiplostomum seoulensis*. *The Korean Journal of Parasitology* **33**, 399.
- Hong, S.T., Yu, J.R., Myong, N.H., Chai, J.Y. and Lee, S.H. (1990). Activities of brush border membrane bound enzymes of the small intestine in *Metagonimus yokogawai* infection in mice. *The Korean Journal of Parasitology* **29**, 9–20.
- Horak, I.G. (1967). Host parasite relationships of *Paramphistomum microbothrium* in experimentally infected ruminants with particular reference to sheep. *Ondestepoort Journal of Veterinary Research* **34**, 451–540.
- Horak, I.G. (1971). Paramphistomiasis of domestic ruminants. *Advances in Parasitology* **9**, 33–70.
- Horak, I.G. and Clark, R. (1963). Studies on paramphistomiasis. V. The pathological physiology of the acute disease in sheep. *Ondestepoort Journal of Veterinary Research* **30**, 145–160.
- Hosier, D.W. and Fried, B. (1991). Infectivity, growth, and distribution of *Echinostoma caproni* (Trematoda) in the ICR mouse. *Journal of Parasitology* **77**, 640–642.
- Hosier, D.W., Fried, B. and Szewczak, J.P. (1988). Homologous and heterologous resistance of *Echinostoma revolutum* and *E. liei* in ICR mice. *Journal of Parasitology* **74**, 89–92.
- Huffman, J.E. (2000). Echinostomes in veterinary and wildlife parasitology. In: *Echinostomes as Experimental Models for Biological Research* (B. Fried and T.K. Graczyk, eds), pp. 59–82. Dordrecht: Kluwer Academic Publishers.
- Huffman, J.E. and Fried, B. (1990). *Echinostoma* and Echinostomiasis. *Advances in Parasitology* **29**, 215–269.
- Huffman, J.E., Fried, B., Roscoe, D.E. and Cali, A. (1984). Comparative pathologic features and development of *Sphaeridiotrema globulus* (Trematoda) in the mute swan and domestic chicken and chicken chorioallantois. *American Journal of Veterinary Research* **45**, 387–391.
- Huffman, J.E., Iglesias, D. and Fried, B. (1988). *Echinostoma revolutum*: pathology of extraintestinal infection in the golden hamster. *International Journal for Parasitology* **18**, 873–874.
- Huffman, J.E., Michos, C. and Fried, B. (1986). Clinical and pathological effects of *Echinostoma revolutum* (Digenea: Echinostomatidae) in the golden hamster, *Mesocricetus auratus*. *Parasitology* **93**, 505–515.
- Huffman, J.E., Sabol, C. and Fried, B. (1991). Infectivity, growth, survival, and pathogenicity of *Zygoctyle lunata* (Trematoda) in experimental rodent hosts. *Journal of Parasitology* **77**, 280–284.
- Huh, S., Chai, J.Y., Hong, S.T. and Lee, S.H. (1988). Clinical and histopathological findings in mice heavily infected with *Fibricola seoulensis*. *The Korean Journal of Parasitology* **26**, 45–53.

- Huh, S. and Hong, S.T. (1993). Transmission electron microscopic findings of the tribocytic organ of *Fibricola seoulensis*. *The Korean Journal of Parasitology* **31**, 315–320.
- Huh, S., Lee, S.H. and Seo, B.S. (1990). Histochemical findings of the tribocytic organ and tegument of *Fibricola seoulensis*. *The Korean Journal of Parasitology* **28**, 155–160.
- Huh, A., Lee, S.U., Won, M.H., Jeong, Y.G., Kwon, Y.H. and Choi, C.S. (1995). IgA response in mice infected with *Neodiplostomum seoulensis*. *The Korean Journal of Parasitology* **33**, 55–60.
- Ibarra, F., Montenegro, N., Vera, Y., Boulard, C., Quiroz, H., Flores, J. and Ochoa, P. (1998). Comparison of three ELISA tests for seroepidemiology of bovine fascioliosis. *Veterinary Parasitology* **77**, 229–236.
- Isaacson, A.C., Huffman, J.E. and Fried, B. (1989). Infectivity, growth, development and pathology of *Echinostoma caproni* (Trematoda) in the golden hamster. *International Journal for Parasitology* **19**, 943–944.
- Jang, Y.K., Kang, S.Y., Kim, S.I. and Cho, S.Y. (1985). In situ posture of anterior body of *Metagonimus yokogawai* in experimentally infected dog. *The Korean Journal of Parasitology* **23**, 203–213.
- Jaroonvesama, N., Charoenlarp, K. and Areekul, S. (1986). Intestinal absorption studies in *Fasciolopsis buski* infection. *Southeast Journal of Tropical Medicine and Public Health* **17**, 582–586.
- Jones, A. (2005). Family Paramphistomidae Fischöder, 1909. In: *Keys to the Trematoda* (D.I. Gibson, A. Jones and R.A. Bray, eds), Vol. 2, pp. 229–246. Wallingford: CABI Publishing.
- Kamiya, H. and Machida, M. (1977). *Brachylaima ishigakiense* n. sp. (Trematoda, Brachylaimidae) from roof rat. *Bulletin of the National Science Museum, Tokyo, Ser. A, Zoology* **3**, 125–129.
- Kang, S.Y., Cho, S.Y., Chai, J.Y., Lee, J.B. and Jang, D.H. (1983). A study on intestinal lesions of experimentally reinfected dogs with *Metagonimus yokogawai*. *The Korean Journal of Parasitology* **21**, 53–73.
- Khan, W.I. and Collins, S.M. (2004). Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. *Parasite Immunology* **26**, 319–326.
- Kho, W.G., Chai, J.Y., Chun, C.H. and Lee, S.H. (1990). Mucosal mast cell responses to experimental *Fibricola seoulensis* infection in rats. *Seoul Journal of Medicine* **31**, 191–199.
- Kim, S.I. (1998). A *Clonorchis sinensis*-specific antigen that detects active human clonorchiasis. *The Korean Journal of Parasitology* **36**, 37–45.
- Kim, S. and Fried, B. (1989). Pathological effects of *Echinostoma caproni* (Trematoda) in the domestic chick. *Journal of Helminthology* **63**, 227–230.
- Kim, I., Im, J.A., Lee, K.J. and Ryang, Y.S. (2000). Mucosal mast cell responses in the small intestine of rats infected with *Echinostoma hortense*. *The Korean Journal of Parasitology* **38**, 139–143.

- Kishore, N. and Sinha, D.P. (1982). Observations on *Echinostoma revolutum* infection in the rectum of domestic chicks (*Anas platyrhynchos domestica*). *Agricultural Science Digest* **2**, 57–60.
- Kook, J., Nawa, Y., Lee, S.H. and Chai, J.Y. (1998). Pathogenicity and lethality of a minute intestinal fluke, *Neodiplostomum seoulense*, to various strains of mice. *Journal of Parasitology* **84**, 1178–1183.
- Kostadinova, A. (2005). Family Echinostomatidae Looss, 1899. In: *Keys to the Trematoda* (D.I. Gibson, A. Jones and R.A. Bray, eds), Vol. 2, pp. 9–64. Wallingford: CABI Publishing.
- Kostadinova, A. and Gibson, D. (2000). The systematics of the Echinostomes. In: *Echinostomes as Experimental Models for Biological Research* (B. Fried and T.K. Graczyk, eds), pp. 31–57. Dordrecht: Kluwer Academic Publishers.
- Kostadinova, A., Gibson, D.I., Biserkov, V. and Chipev, N. (2000). Re-validation of *Echinostoma miyagawai* Ishii, 1932 (Digenea: Echinostomatidae) on the basis of the experimental completion of its life-cycle. *Systematic Parasitology* **45**, 81–108.
- Kramer, M.H., Eberhard, M.L. and Blankenberg, T.A. (1996). Respiratory symptoms and subcutaneous granuloma caused by mesocercariae: a case report. *American Journal of Tropical Medicine and Hygiene* **55**, 447–448.
- Kumari, P.S. and Hafeez, M. (2004). Immuno-electrophoresis of paramephistomosis in cattle. *Indian Journal of Animal Sciences* **74**, 499–500.
- Lantz, C.S., Boesiger, J., Song, C.H., Mach, N., Kobayashi, T., Mulligan, R.C., Nawa, Y., Dranoff, G. and Galli, S.J. (1998). Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature* **392**, 90–93.
- Lawrence, C.E. (2003). Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunology* **25**, 271–281.
- Lee, S.H. and Chai, J.Y. (2001). A review of *Gymnophalloides seoi* (Digenea: Gymnophallidae) and human infections in the Republic of Korea. *The Korean Journal of Parasitology* **39**, 85–118.
- Lee, S.H., Chai, J.Y. and Hong, S.T. (1993a). *Gymnophalloides seoi* n. sp. (Digenea: Gymnophallidae), the first report of human infection by a gymnophallid. *The Korean Journal of Parasitology* **79**, 677–680.
- Lee, S.H., Chai, J.Y., Seo, M., Choi, M.H., Kim, D.C. and Lee, S.K. (1995). Two cases of *Gymnophalloides seoi* infection accompanied by diabetes mellitus. *The Korean Journal of Parasitology* **33**, 61–64.
- Lee, J.B., Chi, J.G., Lee, S.K. and Cho, S.Y. (1981). Study on the pathology of metagonimiasis in experimentally infected cat intestine. *The Korean Journal of Parasitology* **19**, 109–129.
- Lee, S.C., Chung, Y.B., Kong, Y., Kang, S.Y. and Cho, S.Y. (1993b). Antigenic protein fractions of *Metagonimus yokogawai* reacting with patient sera. *The Korean Journal of Parasitology* **31**, 43–48.

- Lee, S.H., Kim, B.I., Hong, S.T., Sohn, W.M. and Chai, J.Y. (1989). Observation of mucosal pathology after praziquantel treatment in experimental *Fibricola seoulensis* infection in rats. *The Korean Journal of Parasitology* **27**, 35–40.
- Lee, J.C., Kong, Y., Lee, S.U. and Huh, S. (1997a). Localization of worm antigen in *Neodiplostomum seoulense* by immuno-electronmicroscopy. *The Korean Journal of Parasitology* **35**, 95–104.
- Lee, K.J., Park, S.K., Im, J.A., Kim, S.K., Kim, G.H., Kim, G.Y., Yang, E.J. and Ryang, Y.S. (2004). Susceptibility of several strains of mice to *Echinostoma hortense* infection. *The Korean Journal of Parasitology* **42**, 51–56.
- Lee, S.H., Park, S.K., Seo, M., Guk, S.M., Choi, M.H. and Chai, J.Y. (1997b). Susceptibility of various species of animals and strains of mice to *Gymnophalloides seoi* infection and the effects of immunosuppression in C3H/HeN mice. *Journal of Parasitology* **83**, 883–886.
- Lee, S.H., Yoo, B.H., Hong, S.T., Chai, J.Y., Seo, B.S. and Chi, J.G. (1985). A histopathological study on the intestine of mice and rats experimentally infected by *Fibricola seoulensis*. *The Korean Journal of Parasitology* **23**, 58–72.
- Loukas, A. and Prociv, P. (2001). Immune responses in hookworm infections. *Clinical Microbiology Reviews* **14**, 689–703.
- Mabus, J., Huffman, J.E. and Fried, B. (1988). Humoral and cellular response to infection with *Echinostoma revolutum* in the golden hamster. *Mesocricetus auratus*. *Journal of Helminthology* **62**, 127–132.
- MacGlashan, D., Jr. (2003). Histamine: a mediator of inflammation. *Journal of Allergy and Clinical Immunology* **112**, 53–59.
- Maizels, R.M., Bundy, D.A., Selkirk, M.E., Smith, D.F. and Anderson, R.M. (1993). Immunological modulation and evasion by helminth parasites in human populations. *Nature* **365**, 797–805.
- Maizels, R.M. and Holland, M.J. (1998). Pathways for expelling intestinal helminths. *Current Biology* **8**, 711–714.
- Maizels, R.M. and Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology* **3**, 733–744.
- Maji, B.P., Dwivedi, P. and Rao, J.R. (1997a). Assessment of antigenic cross-reactivity among rumenal and biliary paramphistomes by Ouchterlony double immunodiffusion test. *Journal of Veterinary Parasitology* **11**, 47–50.
- Maji, B.P., Dwivedi, P. and Rao, J.R. (1997b). Polypeptides of rumenal and biliary paramphistomes analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. *Journal of Veterinary Parasitology* **11**, 165–168.
- Maji, B.P., Dwivedi, P. and Rao, J.R. (1998). Immunoelectrophoretic analysis of paramphistomid antigens. *Indian Journal of Animal Sciences* **68**, 1–3.

- Maji, B.P., Dwivedi, P., Rao, J.R. and Yadav, S.C. (1999). Cross-antigenicity between ruminal and hepatic paramphistomes and liver flukes of buffalo origin. *Journal of Applied Animal Research* **16**, 53–57.
- Marcilla, A., Rubia, J.E., Espert, A., Carpena, I., Esteban, J.G. and Toledo, R. (2004). Specific tyrosine phosphorylation in response to bile in *Fasciola hepatica* and *Echinostoma friedi*. *Experimental Parasitology* **106**, 56–58.
- Martínez-Alonso, J.C., Armentia, A., Vega, J.M., Callejo, A. and Gomez, A. (1999). Anaphylactic reaction concurrent with *Heterophyes heterophyes* infestation. *Revista Española de Alergología e Inmunología Clínica* **14**, 37–39.
- Marty, A.M. and Andersen, E.M. (2000). Fasciolopsiasis and other intestinal trematodiasis. In: *Pathology of Infectious Diseases. Vol. I Helminthiasis* (W.M. Meyers, R.C. Neafie, A.M. Marty and D.J. Wear, eds), pp. 93–105. Washington, DC: Armed Forces Institute of Pathology, American Registry of Pathology.
- McDonald, H.R., Kazakos, K.R., Schatz, H. and Johnson, R.N. (1994). Two cases of intraocular infection with *Alaria mesocercariae* (Trematoda). *American Journal of Ophthalmology* **117**, 447–455.
- Mucha, K.H. and Huffman, J.E. (1991). Inflammatory cell stimulation and wound healing in *Sphaeridotrema globulus* experimentally infected mallard ducks (*Anas platyrhynchos*). *Journal of Wildlife Diseases* **27**, 428–434.
- Mulcahy, G., O'Neill, S., Donnelly, S. and Dalton, J.P. (2004). Helminths at mucosal barriers –interaction with the immune system. *Advanced Drug Delivery Reviews* **56**, 853–868.
- Muñoz-Antoli, C., Carpena, I., Espert, A., Esteban, J.G. and Toledo, R. (2004). The effect of host species on the development of *Echinostoma friedi* (Trematoda: Echinostomatidae) adult worms. *Revista Ibérica de Parasitología* **64**, 81–87.
- Nawa, Y., Ishikawa, N., Tsuchiya, K., Horii, Y., Abe, T., Khan, A.I., Bing-Shi, Itoh, H., Ide, H. and Uchiyama, F. (1994). Selective effector mechanisms for the expulsion of intestinal helminths. *Parasite Immunology* **16**, 333–338.
- Niewiadomska, K. (2002). Family Diplostomidae Poirier, 1886. In: *Keys to the Trematoda* (D.I. Gibson, A. Jones and R.A. Bray, eds), Vol. 1, pp. 167–196. Wallingford: CABI Publishing.
- Odaibo, A.B., Christensen, N.Ø. and Ukoli, F.M.A. (1988). Establishment, survival and fecundity in *Echinostoma caproni* (Trematoda) infections in NMRI mice. *Proceedings of the Helminthological Society of Washington* **55**, 265–269.
- Odaibo, A.B., Christensen, N.Ø. and Ukoli, F.M.A. (1989). Further studies on the population regulation in *Echinostoma caproni* infections in NMRI mice. *Proceedings of the Helminthological Society of Washington* **56**, 192–198.

- Pica, R., Castellano, C., Cilia, C. and Errico, F.P. (2003). Intestinal fluke infections: the heterophyids. *La Clinica Terapeutica* **154**, 61–63.
- Rho, I.H., Kim, S.I., Kang, S.Y. and Cho, S.Y. (1984). [Observation on the pathogenesis of villous changes in early phase of experimental metagonimiasis] (in Korean). *Chung-Ang Journal of Medicine* **9**, 67–77.
- Rim, H.J., Kim, S.J. and Yang, M.G. (1992). [Antigenic localities in the tissues of *Metagonimus yokogawai* in the period of growth] (in Korean). *The Korean Journal of Parasitology* **30**, 309–321.
- Rolfe, P.F., Boray, J.C. and Collins, G.H. (1994). Pathology of infection with *Paramphistomum ichikawai* in sheep. *International Journal for Parasitology* **24**, 995–1004.
- Roscoe, D.E. and Huffman, J.E. (1982). Trematode (*Sphaeridotrema globules*)-induced ulcerative hemorrhagic enteritis in wild mute swans (*Cygnus olor*). *Avian Diseases* **26**, 214–224.
- Roscoe, D.E. and Huffman, J.E. (1983). Fatal enteritis caused by *Sphaeridotrema globulus* (Trematoda: Psilostomidae) in whistling swan. *Journal of Wildlife Diseases* **19**, 370–371.
- Ryang, Y.S., Yoo, J.C., Lee, S.H. and Chai, J.Y. (2001). Susceptibility of avian hosts to experimental *Gymnophalloides seoi* infection. *Journal of Parasitology* **87**, 454–456.
- Sahai, B.N., Singh, R.P. and Prasad, G. (1985). Histochemical alterations in the duodenum of goats experimentally infected with *Paramphistomum cervi*. *Veterinary Parasitology* **17**, 131–138.
- Saifullah, M.K., Ahmad, G. and Nizami, W.A. (2000a). Analysis of excretory-secretory and somatic antigens of *Gastrothylax crumenifer*. *Journal of Helminthology* **74**, 271–276.
- Saifullah, M.K., Ahmad, G., Nizami, W.A. and Abidi, S.M.A. (2000b). Partial purification and characterization of *Gastrothylax crumenifer* somatic antigens. *Veterinary Parasitology* **89**, 23–29.
- Scholz, T. (2002). Family Gymnophallidae Odhner, 1905. In: *Keys to the Trematoda* (D.I. Gibson, A. Jones and R.A. Bray, eds), Vol. 1, pp. 245–251. Wallingford: CABI Publishing.
- Seo, M., Guk, S.M., Han, E.T. and Chai, J.Y. (2003). Role of intestinal goblet cells in the expulsion of *Gymnophalloides seoi* from mice. *Journal of Parasitology* **89**, 1080–1082.
- Seo, B.S., Lee, S.H., Chai, J.Y., Hong, S.J. and Hong, S.T. (1988). The life cycle and larval development of *Fibricola seoulensis* (Trematoda: Diplostomatidae). *The Korean Journal of Parasitology* **26**, 179–188.
- Seo, B.S., Lee, S.H., Hong, S.T., Hong, S.J., Kim, C.Y. and Lee, H.Y. (1982). Studies on intestinal trematodes in Korea: V. A human case infected by *Fibricola seoulensis* (Trematoda: Diplostomatidae). *The Korean Journal of Parasitology* **20**, 93–99.

- Seo, B.S., Rim, H.J. and Lee, C.W. (1964). Studies on the parasitic helminths of Korea: I Trematodes of rodents. *The Korean Journal of Parasitology*, **2**, 20–26.
- Sey, O. (1991). *CRC Handbook of the Zoology of Amphistomes*, p. 480. Florida, USA: CRC Press.
- Sharma-Deorani, V.P. and Katiyar, R.D. (1967). Studies on the pathogenicity due to immature amphistomes among sheep and goats. *Indian Veterinary Journal* **44**, 199–205.
- Shin, E.H., Chai, J.Y. and Lee, S.H. (2001). Extraintestinal migration of *Pharyngostomum cordatum* metacercariae in experimental rodents. *Journal of Helminthology* **75**, 285–290.
- Shin, E.H., Kim, T.H., Hong, S.J., Park, J.H., Guk, S.M. and Chai, J.Y. (2003). Effects of anti-allergic drugs on intestinal mastocytosis and worm expulsion of rats infected with *Neodiplostomum seoulense*. *The Korean Journal of Parasitology* **41**, 81–87.
- Shoop, W.L. (1989). Experimental human infection with *Fibricola cratera* (Trematoda: Neodiplostomidae). *The Korean Journal of Parasitology* **27**, 249–252.
- Shoop, W.L. and Corkum, K.C. (1984). Transmammary infection of newborn by larval trematodes. *Science* **223**, 1082–1083.
- Simonsen, P.E. and Andersen, B.J. (1986). *Echinostoma revolutum* in mice: dynamics of the antibody attack to the surface of an intestinal trematode. *International Journal for Parasitology* **16**, 475–482.
- Simonsen, P.E., Bindseil, E. and Koie, M. (1989). *Echinostoma caproni* in mice: studies on the attachment site of an intestinal trematode. *International Journal for Parasitology* **19**, 561–566.
- Simonsen, P.E., Estambale, B.B. and Agger, M. (1991). Antibodies in the serum of golden hamsters experimentally infected with the intestinal trematode *Echinostoma caproni*. *Journal of Helminthology* **65**, 239–247.
- Simonsen, P.E., Vennervald, B.J. and Birch-Andersen, A. (1990). *Echinostoma caproni* in mice: ultrastructural studies on the formation of immune complexes on the surface of an intestinal trematode. *International Journal for Parasitology* **20**, 935–941.
- Singh, C.D.N. and Lakra, P. (1971). Pathologic changes in naturally occurring *Cotylophoron cotylophorum* infection in cattle. *American Journal of Veterinary Research* **32**, 659.
- Singh, R.P., Sahai, B.N. and Jha, G.J. (1984). Histopathology of the duodenum and rumen of goats during experimental infections with *Paraphistomum cervi*. *Veterinary Parasitology* **15**, 39–46.
- Sirag, S.B., Christensen, N.O., Frandsen, F., Monrad, J. and Nansen, P. (1980). Homologous and heterologous resistance in *Echinostoma revolutum* infections in mice. *Parasitology* **80**, 479–486.

- Snyder, D.E., Hamir, A.N., Hanlon, C.A. and Rupprecht, C.E. (1989). *Phagicola angrense* (Digenea: Heterophyidae) as a cause of enteritis in a raccoon (*Procyon lotor*). *Journal of Wildlife Diseases* **25**, 273–275.
- Tani, S. and Yoshimura, K. (1988). Spontaneous expulsion of *Echinostoma hortense* Asada, 1926 (Trematoda: Echinostomatidae) in mice. *Parasitology Research* **74**, 495–497.
- Thorndyke, M.C. and Whitfield, P.J. (1987). Vasoactive intestinal polypeptide-like immunoreactive tegumental cells in the digenean helminth *Echinostoma liei*, possible role in host-parasite interactions. *General and Comparative Endocrinology* **68**, 202–207.
- Toledo, R., Espert, A., Carpena, I., Muñoz-Antoli, C. and Esteban, J.G. (2003a). An experimental study of the reproductive success of *Echinostoma friedi* (Trematoda: Echinostomatidae) in the golden hamster. *Parasitology* **126**, 433–441.
- Toledo, R., Espert, A., Carpena, I., Muñoz-Antoli, C., Fried, B. and Esteban, J.G. (2004a). The comparative development of *Echinostoma caproni* (Trematoda: Echinostomatidae) adults in experimentally infected hamsters and rats. *Parasitology Research* **93**, 439–444.
- Toledo, R., Espert, A., Muñoz-Antoli, C., Fried, B. and Esteban, J.G. (2004c). Immunological characterization of somatic and excretory secretory antigens of *Echinostoma caproni* (Trematoda: Echinostomatidae) in experimentally infected rats. *Comparative Parasitology* **71**, 42–48.
- Toledo, R., Espert, A., Muñoz-Antoli, C., Marcilla, A., Fried, B. and Esteban, J.G. (2003b). Development of an antibody-based capture enzyme-linked immunosorbent assay for detecting *Echinostoma caproni* (Trematoda) in experimentally infected rats: kinetics of coproantigen excretion. *Journal of Parasitology* **89**, 1227–1231.
- Toledo, R., Espert, A., Muñoz-Antoli, C., Marcilla, A., Fried, B. and Esteban, J.G. (2004b). Kinetics of *Echinostoma caproni* (Trematoda: Echinostomatidae) antigens in feces and serum of experimentally infected hamsters and rats. *Journal of Parasitology* **90**, 752–758.
- Toledo, R., Espert, A., Muñoz-Antoli, C., Marcilla, A., Fried, B. and Esteban, J.G. (2005). Kinetics of antibodies and antigens in serum of mice experimentally infected with *Echinostoma caproni* (Trematoda: Echinostomatidae). *Journal of Parasitology* **91**, 978–980.
- Toledo, R. and Fried, B. (2005). Echinostomes as experimental models in adult parasite-vertebrate host interactions. *Trends in Parasitology* **21**, 251–254.
- Toledo, R., Muñoz-Antoli, C. and Esteban, J.G. (2000). The life-cycle of *Echinostoma friedi* (Trematoda: Echinostomatidae) in Spain and a discussion on the relationships within the “*revolutum*” group based on cercarial chaetotaxy. *Systematic Parasitology* **45**, 199–217.
- Trouve, S. and Coustau, C. (1998). Differences in adult excretory-secretory products between geographical isolates of *Echinostoma caproni*. *Journal of Parasitology* **84**, 1062–1065.

- Varma, A.K. (1961). Observations on the biology and pathogenicity of *Cotylophoron cotylophorum* (Fischöeder, 1901). *Journal of Helminthology* **35**, 161-168.
- Varma, T.K., Malviya, H.C., Dwivedi, P. and Prasad, A. (1991). Precipitins in *Paramphistomum cervi* infection. *Indian Journal of Animal Sciences* **61**, 680-682.
- Viyanant, V., Upatham, E.S., Sobhon, P., Krailas, D., Ardseungnoen, P. and Anatawa, S. (1997). Development and characterization of monoclonal antibodies against excretory-secretory antigens of *Fasciola gigantica*. *Southeast Asian Journal of Tropical Medicine and Public Health* **28**, 128-133.
- Weinstein, M.S. and Fried, B. (1991). The expulsion of *Echinostoma trivolvis* and retention of *Echinostoma caproni* in the ICR mouse: pathological effects. *International Journal for Parasitology* **21**, 255-257.
- Whitten, L.K. (1955). Paramphistomiasis in sheep. *New Zealand Veterinary Journal* **3**, 144.
- WHO. (1995). Control of foodborne trematode infections. *World Health Organization. Technical Report* **849**, 1-157.
- Yadav, C.L., Saxena, M.K., Ram, H., Garg, R. and Banerjee, P.S. (2003). Detection of cross-reacting proteins in *Fasciola gigantica* and *Paramphistomum epiclitum*. *Indian Veterinary Journal* **73**, 718-720.
- Yamaguti, S. (1958). *Systema helminthum. Vol I. Digenetic Trematodes of Vertebrates*, p. 1575. New York: Interscience Publications.
- Yamaguti, S. (1971). *Synopsis of Digenetic Trematodes of Vertebrates*, p. 1074+349 plates. Tokyo: Keigaku Publishing Company.
- Yamaguti, S. (1975). *A Synoptical Review of the Life Histories of Digenetic Trematodes of Vertebrates: With Special Reference to the Morphology of Their Larval Forms*, p. 590+219 plates. Tokyo: Keigaku Publishing Company.
- Yu, J.R., Hong, S.T., Chai, J.Y. and Lee, S.H. (1995). The effect of reinfection with *Neodiplostomum seoulensis* on the histopathology and activities of brush border membrane bound enzymes in the rat small intestine. *The Korean Journal of Parasitology* **33**, 37-43.
- Yu, S. and Mott, K. (1994). Epidemiology and morbidity of food-borne intestinal trematode infections. *Tropical Diseases Bulletin* **91**, R125-R152.
- Yu, J.R., Myong, N. and Chai, J.Y. (1997). Expression patterns of proliferating cell nuclear antigen in the small intestine of mice infected with *Metagonimus yokogawai* and *Metagonimus* Miyata type. *The Korean Journal of Parasitology* **35**, 239-244.
- Yu, L.C. and Perdue, M.H. (2001). Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. *Immunological Reviews* **179**, 61-73.
- Zuk, M. and McKean, K. (1996). Sex differences in parasite infections: patterns and processes. *International Journal for Parasitology* **26**, 1009-1024.

Systematics and Epidemiology of *Trichinella*

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ABSTRACT

In this review, we describe the current knowledge on the systematics, ecology and epidemiology of *Trichinella* and trichinellosis, and the impact of recent research discoveries on the understanding of this zoonosis. The epidemiology of this zoonosis has experienced important changes over the past two decades, especially with regard to the importance of the sylvatic cycle and the sylvatic species. Outbreaks of trichinellosis due to *Trichinella spiralis* from domestic swine, while still frequent, increasingly are caused by other *Trichinella* spp. infecting hosts such as horses, dogs, wild boars, bears and walruses. The latter revelations have occurred as a result of a series of discoveries on the systematics of *Trichinella* spp., facilitated by new molecular tools. As a consequence, the genus is now composed of two clades, an encapsulated group (five species and three genotypes) and a non-encapsulated one (three species). This has sparked renewed investigations on the host range of these parasites and their epidemiological features. Most dramatic, perhaps, is the recognition that reptiles may also serve as hosts for certain species. This new knowledge base, in addition to having an important relevance for food safety policies and protection measures, is raising important questions on the phylogeny of *Trichinella* spp., the ecological characteristics of the species and their geographic histories. Answers to these questions may have great value for the understanding of the evolutionary biology for other parasitic helminths, and may increase the value of this genus as models for research on parasitism in general.

1. INTRODUCTION

Trichinellosis, the proper term for the human zoonotic disease also known as trichinosis or trichiniasis, is caused by a group of unusual nematodes belonging to the genus *Trichinella*. This zoonosis has had a long and eventful history of scientific investigation and discovery. Because of the existence of several excellent reviews by others of the history, biology and clinical aspects of trichinellosis (Gould, 1970;

Campbell, 1983a, 1991; Nelson 1988), this introduction will only summarize the major epochs that mark the history of the research on and control of *Trichinella*, but instead highlight those landmarks that have had a major impact on our understanding of these parasites and their important biological properties.

The unraveling of the nature of trichinellosis may reach back to antiquity as suggested by historical references to diseases that bear striking similarities to the clinical aspects of *Trichinella* sp. infection. The earliest such case involved a young Egyptian living along the Nile about 1200 BC (Gould, 1970; Campbell, 1983a). There is evidence of human infections even in prehistoric cultures (Owen *et al.*, 2005). The modern history of trichinellosis, however, begins in 1835, with the discovery, by microscopy, of the larval stage of the parasite by James Paget and Richard Owen in London. It was Owen who coined its first name, *Trichina spiralis* (Owen, 1835). Over the next 60 years, new revelations on the parasite's life cycle, epidemiology and clinical diagnosis resulted from research chiefly carried out in Germany. Several important discoveries stand out, perhaps the most important from a public health standpoint is the linkage of *Trichinella spiralis* infection with the human disease and mortality by Friedrich Zenker in 1860. His identification of the form of origin of a deceased 20-year-old housemaid who had died of *T. spiralis* infection and the subsequent epidemiological detective work he performed demonstrated that the source of infection was pork, the first clear evidence of transmission of *T. spiralis* from an animal to man (Nelson, 1988). This was hailed within the medical community as the most important medical advance of the time (Campbell, 1983a). The other related breakthrough was the discovery of the parasite's life cycle, from the investigations of Rudolf Virchow, F. Zenker, Rudolf Leukart and others. Particularly important was the recognition that *Trichinella* sp. was primarily a parasite of animals, and that it existed in both a domestic cycle (e.g. pigs, rodents and pets) and a sylvatic cycle (wild animals) (Kozar, 1970).

Based on these advances in the understanding of the basic biology of *T. spiralis*, a second phase in the history of the zoonosis unfolded: the adoption of control strategies to prevent infection in humans. Because of the relative ease of detecting microscopically the larval stage (trichina) in selected muscles, the inspection of pork at slaughter

was declared feasible and introduced in 1863 into slaughterhouses, first locally in a few areas of Germany, and then, led by R. Virchow, nationally throughout the country in 1866. This ushered in the now worldwide practice of veterinary control over the slaughter of food animals to ensure food safety.

1.1. *Trichinella* as a Model for Basic Research

With the growing understanding of the epidemiology and clinical aspects of trichinellosis, and the ease of maintaining the parasite's life cycle in the laboratory, the study of *Trichinella* spp. throughout the 20th century expanded to basic investigations on its morphology, the physiology and biochemistry of its intracellular parasitic mode, and on the immunological aspects of infection (Larsh, 1975; Despommier, 1983; Stewart, 1983; Britov, 1994; Castro, 1997; Wakelin and Grencis, 1997; Appleton and Romaris, 2001; Romaris and Appleton, 2001; Bruschi, 2002; Wu *et al.*, 2002). The understanding of the basic biology of *Trichinella* spp. has benefited also from the use of these parasites as a model for a broad range of investigations in parasitology on such general topics as immunology and physiology to the scientific interest in what an understanding of its intrinsic biological features might contribute to gaining important insights into parasitism in general.

1.2. History of *Trichinella* Taxonomy

The third major era in the history of trichinellosis might be fairly characterized as a revolution in the systematics of *Trichinella* spp. and the understanding of its ecology and epidemiology. Although Railliet (1896) revised the genus name to *Trichinella*, inasmuch as the designation *Trichina* had been employed for a genus of Diptera (*Trichina clavipes* Meigen, 1830) (Gould, 1970), important misunderstandings of its taxonomy, host range and epidemiology persisted until well into the 20th century. During the first 150 years of its scientific recognition, *T. spiralis* was considered the sole member of the genus, and as having a phenomenally wide host range, extending to more than 100 species of mammals (Campbell, 1983b). However, beginning in 1950s

and 1960s, scientists began reporting an increasing number of host-specificity peculiarities among different geographic isolates (Rausch *et al.*, 1956; Nelson *et al.*, 1966). Several investigators reported that isolates from some wild animals appeared to have poor infectivity in pigs and rats, the major hosts for the domestic cycle, leading to speculation that important geographic variability existed within the species. Among the earliest reports were those of Rausch *et al.* (1956) and Rausch (1970) that attempts to infect a pig with infected meat from an arctic fox (*Alopex lagopus*) in Alaska were unsuccessful. The breakthrough discovery, however, occurred in Kenya in the 1960s, when Nelson and coworkers convincingly demonstrated that an isolate from the bushpig (*Potamochoerus porcus*), which had been responsible for a human outbreak, had low infectivity in rats and pigs, in comparison to porcine isolates from Europe and the United States (Nelson *et al.*, 1966). These comparative infection results sparked great interest among investigators and led to similar comparative studies with various geographic isolates of the parasite (reviewed by Rausch, 1970). These studies, conducted over the next 30 years, yielded a remarkable series of new revelations on the genetic diversity within the genus, and yielded finally a new *Trichinella* taxonomy encompassing eight species (see Table 1), along with a more complete zoogeographical and epidemiological knowledge base (Pozio *et al.*, 1992a; Murrell *et al.*, 2000; Pozio and Zarlenga, 2005). These advances and the insights they have provided are a focus of this review.

The epidemiological view of trichinellosis has, because of these new biological revelations, expanded from a primarily domestic cycle source to an increasingly sylvatic one (Dupouy-Camet, 2000; Murrell and Pozio, 2000; Pozio, 2000, 2001a). While the zoonosis is continuing to be brought under control in some regions (e.g. Europe and North America), its potential for rebounding, due to laxity in veterinary control, is great because of the parasite's ability to exploit new opportunities for transmission presented by changes in demography, agriculture and wildlife habitat (Boireau *et al.*, 2000; Pozio *et al.*, 2001a, 2005a; Djordevic *et al.*, 2003). Current trends in those factors that have the potential to perturb the ecology and epidemiology of *Trichinella* sp. and their potential consequences are discussed.

Table 1 Main features of *Trichinella* species and genotypes recognised so far

Clade Species or genotype	Geographical distribution	Host range	Main source of infection for humans	Resistance of larvae in frozen muscles
<hr/>				
Encapsulated				
<i>T. spiralis</i>	Cosmopolitan	Domestic and sylvatic mammals	Domestic and sylvatic swine horse	No
<i>T. nativa</i>	Arctic and subarctic areas of the Nearctic and Palearctic regions	Sylvatic carnivores	Bear, walrus	Yes in carnivore muscles
<i>Trichinella</i> T6	Canada; Alaska, Rocky Mountains and Appalachian in the USA	Sylvatic carnivores	Carnivores	Yes in carnivore muscles
<i>T. britovi</i>	Temperate areas of the Palearctic region, Northern and Western Africa	Sylvatic mammals, seldom domestic pigs	Wild boar, domestic pig	Yes in carnivore muscles
<i>Trichinella</i> T8	South Africa and Namibia	sylvatic carnivores	Non-documented	No
<i>T. murrelli</i>	USA and Southern Canada	Sylvatic carnivores	Bear, horse	No
<i>Trichinella</i> T9	Japan	Sylvatic carnivores	Non-documented	No
<i>T. nelsoni</i>	Eastern-Southern Africa	Sylvatic mammals	Warthog, bush pig	No
<hr/>				
Non-encapsulated				
<i>T. pseudospiralis</i>	Cosmopolitan	Sylvatic mammals and birds, domestic pigs	Domestic and wild pigs	No
<i>T. papuae</i>	Papua New Guinea	Wild pig, saltwater crocodile	Wild pig	No
<i>T. zimbabwensis</i>	Zimbabwe, Mozambique, Ethiopia	Nile crocodile, monitor lizard	Non-documented	No

2. ADVANCES IN THE SYSTEMATICS OF *TRICHINELLA*

The genus *Trichinella* is the sole member of the Family: Trichinellidae Ward, 1907, Order: Trichocephalida, Class: Nematoda, Phylum: Nematelminthes. According to Blaxter *et al.* (1998), this Order is an archaic group, related to such basal nematodes as the free-living Mononchida, the plant parasitic Dorylamida and the entomophagous Mermithida. As mentioned above, it was eventually realized that different isolates of *T. spiralis* possessed important biological differences, even though no reliable morphological features could be identified. However, following the pioneer studies of Nelson and Mukundi (1963), Kozar and Kozar (1965), Perevertseva (1966), Britov (1969) and Rausch (1970), two different experimental approaches evolved for characterizing various isolates: (1) comparison of reproductive potentials, i.e. the reproductive capacity index or RCI, which is the ratio of the number of recovered larvae to the number of larvae administered to laboratory rodents (Kruger *et al.*, 1969; Arakawa and Todd, 1971), and (2) the ability of two different isolates to interbreed in laboratory mice (usually reciprocal parasite gender mating) (Britov, 1971). Based on the results from these experimental approaches, particularly interbreeding experiments, Britov and Boev (1972) described two new species of *Trichinella*: *T. nativa*, which was widespread among wildlife of the arctic and sub-arctic regions; and *T. nelsoni*, considered to be widely distributed in the temperate areas of the Palearctic region and in Africa. Simultaneously, a third new species, *T. pseudospiralis*, was described by Garkavi (1972) in the Caucasus from a raccoon (*Procyon lotor*), mainly on the basis of a lack of a collagen capsule around the larva in the muscle cell and the larva's smaller size, a property lacking for the other species. The description of these species generated an intense debate over their taxonomic validity, however, because of the lack of clear morphological differences among these proposed species (Lichtenfels *et al.*, 1983). Soon, however, *T. pseudospiralis* (Garkavi's strain) became widespread in laboratories throughout the world as a reference strain for this species, and for comparative studies, principally with *T. spiralis*, which generated considerable new comparative biological data that provided persuasive evidence for its species validity. Much of the controversy over these new

species revolved around questions of the validity of the interbreeding method for detecting genetic incompatibility, and the lack of a consensus on the definition of species (see Dick, 1983). Although there was little disagreement that there were important biological differences among various geographical and host species isolates, the debate revolved mainly around their appropriate taxonomic position (whether to classify them as ecotypes, subspecies or species).

2.1. Biochemical and Molecular Studies

The resolution of the taxonomic issues has only occurred in the last 15 years, facilitated by the adoption of new biochemical and molecular techniques for systematics research. Initial attempts to apply biochemical methods to investigate genetic variation among *Trichinella* isolates revealed consistent allozyme differences between certain isolates with different host and geographical origins (Flockhart *et al.*, 1982). Allozyme patterns were then produced for a large number of isolates, confirming the existence of consistent genetic variability among certain isolates, even if the lack of formal types of the existed isolates prevented a general agreement and a clear comprehension of the taxonomic status of this nematode genus (Mydyski and Dick, 1985; Fukumoto *et al.*, 1987, 1988; Murrell *et al.*, 1987a; Pozio, 1987). Early molecular tools based on parasite DNA also readily distinguished these particular isolates, supporting the concept that the genus *Trichinella* was genetically complex, and that the genetic differences marked important biological variation (Chambers *et al.*, 1986; Dame *et al.*, 1987; Zarlenga and Barta, 1990; Zarlenga and Dame, 1992).

The first comparative analysis of biochemical differences among a large number of isolates (152) appeared in 1992, the results of which proved to be a landmark in the clarification of the systematics of *Trichinella* (La Rosa *et al.*, 1992). The study, comparing 27 allozyme patterns of 152 isolates from various host species and geographical regions, identified eight distinct genotypes (with the code from T1 to T8), four of which represented the four previously proposed species (*T. spiralis*, *T. nativa*, *T. nelsoni* and *T. pseudospiralis*) (La Rosa *et al.*, 1992). This study also compared seven biological and two

morphological characters of 40 of the isolates, and the results supported the recognition of the same eight genotypes (Pozio *et al.*, 1992b). The meta-analysis of these data and data from the published literature, encompassing about 300 *Trichinella* isolates, formed the basis for a taxonomic revision of the genus, in which five sibling species were recognized: *T. spiralis*, *T. nativa* and *T. pseudospiralis*, as previously proposed, *T. nelsoni*, restricted to the isolates from tropical Africa, and *T. britovi* n. sp. for the Palearctic isolates previously misidentified as *T. nelsoni sensu stricto* (Britov and Boev, 1972). In addition, three genotypes, named *Trichinella* T5, T6 and T8, whose taxonomic rank was unclear, were also identified (Pozio *et al.*, 1992a). This new taxonomic scheme, which has remained the paradigm up to the present, has only been modified to add new species (Table 1) (Murrell *et al.*, 2000; Pozio and Zarlenga, 2005).

2.2. The Polymerase Chain Reaction Era

The introduction of polymerase chain reaction (PCR)-derived method has proved important in simplifying the identification of *Trichinella* isolates from different host species and geographical regions and has confirmed the current taxonomy of the genus (Table 1) (Dick *et al.*, 1992; Bandi *et al.*, 1993, 1995; Soule *et al.*, 1993; Wang *et al.*, 1995; Appleyard *et al.*, 1999; Nagano *et al.*, 1999; Zarlenga *et al.*, 1999; Gasser *et al.*, 1998, 2004; Wu *et al.*, 1998, 1999, 2000; Rombout *et al.*, 2001; Pozio and La Rosa, 2003). Its greater sensitivity has also permitted the opportunity to analyze single larvae, which revealed the occurrence of mixed species infections in the same host, a finding that has added important information on the potential for gene flow between sympatric species or genotypes (Pozio *et al.*, 1995; 1997a; Malakauskas, 2002; Oivanen *et al.*, 2002). In 1999, a new species, *T. papuae* was identified in Papua New Guinea on the basis of its molecular and biological characteristics (Pozio *et al.*, 1999a). In 2000, the genotype *Trichinella* T5 was erected to the species level as *T. murrelli*, chiefly on the basis of interbreeding experiments (Pozio and La Rosa, 2000), attesting to the value that biological analyses can continue to have in resolving molecular uncertainties. In 2002, a new species, *T. zimbabwenis*, the first to be recognized as

infecting reptiles, was identified on the basis of biological, biochemical and molecular data (Pozio *et al.*, 2002).

A major resource that provided vital parasite material and information for these studies is the International *Trichinella* Reference Centre (ITRC), established in 1990, and which now contains more than 1600 isolates from around the world. Each isolate includes, in addition to its molecular typing results, data on their host species, geographical origin, and other epidemiological information (Pozio *et al.*, 1989, 2001b; www.iss.it/site/Trichinella/index.asp).

2.3. Current Methods for *Trichinella* spp. identification

Except for the existence of some size differential in *T. pseudospiralis*, all species and genotypes of the genus *Trichinella* are morphologically indistinguishable at all developmental stages (new born larvae, muscle larvae and adults). Consequently, only biochemical or molecular methods can be used reliably to identify the species or the genotype.

The use of allozymes as markers has been replaced over time by DNA methods because the latter requires so much less parasite material, and the reduced need to produce large amounts of parasite in laboratory animals, a practice that risks the loss of genetic variability. However, the allozyme analysis is still useful for phylogenetic studies (La Rosa *et al.*, 2003a).

There are a large number of molecular methods useful for identifying species and genotypes, including (1) repetitive DNA probes (Klassen *et al.*, 1986; Dame *et al.*, 1987; La Rosa *et al.*, 1994); (2) restriction fragment length polymorphism (RFLP) (Zarlenga *et al.*, 1991; Nagano *et al.*, 1999); and (3) random amplified polymorphic DNA (RAPD)-derived primers (Wu *et al.*, 1998, 1999). Among PCR-derived methods the RAPD-PCR (Bandi *et al.*, 1993, 1995) has demonstrated low reproducibility, even though it permits the detection and identification of a single muscle larva. Other methods with much higher sensitivity and specificity (multiplex PCR, PCR-RFLP, PCR-single strand conformational polymorphism and reverse line blot hybridization) are also capable of identifying a single larva and consequently are routinely used in laboratories throughout the world (Table 2).

Table 2 Polymerase chain reaction derived methods to identify species and genotypes of single larvae of *Trichinella*

Molecular marker	PCR method	Identified species and genotypes ¹	References
Mitochondrial cytochrome c-oxidase subunit I gene	PCR-RFLP	Ts, Tna, Tb, Tp, Tm, Tne, T6, T8, T9	Nagano <i>et al.</i> , 1999
ITS1, ITS2, ES ²	Multiplex-PCR	Ts, Tna, Tb, TpN, TpP, TpA, Tm, Tne, T6	Zarlenga <i>et al.</i> , 1999
SB153 RAPD SB372 RAPD	PCR-RFLP-SSCP	Ts, Tna, Tb, Tp, Tm, T6, T8, T9	Wu <i>et al.</i> , 2000
5S ribosomal DNA	Reverse line blot hybridization	Ts, Tna, Tb, Tp, Tm, Tne, T6, T8	Rombout <i>et al.</i> , 2001
ITS1, ITS2, ES ²	Multiplex-PCR	Ts, Tna, Tb, TpN, TpP, TpA, Tm, Tne, Tpa, Tz, T6	Pozio and La Rosa, 2003
ES ² , D3 domain of nuclear ribosomal DNA	PCR-SSCP	Ts, Tna-T6, ³ Tb, Tp, Tm, Tne, Tpa-Tz ³ , T8	Gasser <i>et al.</i> , 2004
ITS2	PCR	Tb, T8	Pozio <i>et al.</i> , 2005b

¹*Trichinella spiralis*, Ts; *Trichinella nativa*, Tna; *Trichinella britovi*, Tb; *Trichinella pseudospiralis* of the Nearctic region, TpN; *Trichinella pseudospiralis* of the Palearctic region, TpP; *Trichinella pseudospiralis* of the Australian region, TpA; *Trichinella murrelli*, Tm; *Trichinella nelsoni*, Tne; *Trichinella papuae*, Tpa; *Trichinella zimbabwensis*, Tz; *Trichinella* T6, T6; *Trichinella* T8, T8; *Trichinella* T9, T9.

²ES = expansion segment V region of the ribosomal DNA repeat.

³The two taxonomic entities cannot be distinguished between them.

3. THE TAXONOMY OF THE GENUS

Biological, biochemical and molecular data all support the existence of two main clades in the genus *Trichinella*; one that encompasses species which induce a nurse cell to form a thick collagen capsule around the larva in the host muscle tissue (encapsulated), and a second that includes non-encapsulated species which induce a very thin collagen capsule only visible by the electron microscope (Table 1) (Xu *et al.*, 1997; Pozio *et al.*, 2001c; Pozio *et al.*, 2002; La Rosa *et al.*, 2003a; Gasser *et al.*, 2004; Zarlenga *et al.*, 2004). Encapsulated species and genotypes are restricted to mammals, whereas the three non-encapsulated species are more diverse in host range: *T. pseudospiralis* infects both mammals and birds.

and *T. papuae* and *T. zimbabwensis* parasitise mammals and reptiles (Table 1) (Pozio *et al.*, 2004a). Non-encapsulated species reportedly induce a lower immunopathological response in the mouse muscle than do encapsulated species as indicated by a lower number of infiltrating inflammatory cells surrounding the larvae (Stewart, 1989).

3.1. The Encapsulated Clade

Five species and three genotypes of undetermined taxonomic status belong to this mammal-infecting clade.

3.1.1. *Trichinella spiralis* (Owen, 1835) (Genotype T1)

This is the first species discovered and the most characterized because of its importance both as a cause of human disease and as a model for basic biological research investigations, due in large part to its relatively high frequency in domestic and sylvatic animals and to its high infectivity for laboratory animals. This species probably originated in East Asia (see Section 4), where its genetic variability is greater than isolates from other regions (La Rosa G., personal communication). It has probably spread along with domestic pigs and, probably, the brown rat (*Rattus norvegicus*) by the migration of people throughout the globe. Dissemination of the parasite and its hosts was especially facilitated by the European colonization of North, Central and South America, New Zealand, Hawaii and Egypt (Figure 1) from the 16th to 20th centuries. Its low resistance to low environmental temperatures may have inhibited its spread among wildlife living in frigid zones. The current geographic distribution of *T. spiralis* (Figure 1) can be linked to three distinct life cycle patterns: (1) countries where *T. spiralis* is present in domestic, synanthropic and sylvatic animals (i.e. Europe, Egypt, China, Russia, Southeast Asia, Argentina, Chile, Mexico, New Zealand and the United States) (Mason, 1978; Barakat *et al.*, 1982; Khamboonruang 1991; Mikhail *et al.*, 1994; Schenone *et al.*, 1994; Buncic, 1997; Correa *et al.*, 1997; Gasser *et al.*, 1998; Venturiello *et al.*, 1998; Ortega Pierres *et al.*, 2000; Takahashi *et al.*, 2000; Pozio, 2001a, 2001b; Pozio and Zarlenga, 2005; Ribicich *et al.*, 2005); (2) countries



Figure 1 World map showing the distribution area of *Trichinella spiralis*. The distribution is strongly influenced by human activity, which probably passively introduced this zoonotic pathogen into North, Central and South America, New Zealand and Egypt. (Redrawn from [www.iss.it site Trichinella.index.asp](http://www.iss.it/site/Trichinella/index.asp)).

where *T. spiralis* was present in the past in domestic and synanthropic animals but is currently reported only in sylvatic animals (i.e. Austria, the Czech Republic, France, Germany, Hungary, the Slovak Republic, Sweden, the Netherlands and Canada) (Dick and Pozio, 2001; Pozio, 2001a); and (3) countries where *T. spiralis* was never introduced or has been eradicated, i.e. Italy, Portugal and Switzerland and all of Africa except Egypt (Gottstein *et al.*, 1997; Pozio and La Rosa, 1998; Pozio 2001a; Pozio *et al.*, 2005b).

The main biological features of *T. spiralis* in comparison to other species and genotypes are (1) highest RCI in rodents (mice and rats) and swine (both domestic and sylvatic) (Murrell *et al.*, 1985; Pozio *et al.*, 1992b; Yao *et al.*, 1997; Kapel and Gamble, 2000; Kapel, 2001; Malakauskas and Kapel, 2003); (2) the length of the uterus is the longest in comparison to that of *T. nelsoni* and *T. nativa*. The length of uterus has a direct correlation with the infectivity index, i.e. the longer the uterus, more the intrauterine larval capacity and higher the infectivity index (Sukhdeo and Meerovitch, 1977); (3) highest number of newborn larvae produced by female worms (Sukhdeo and Meerovitch, 1977; Boev *et al.*, 1979; Pozio *et al.*, 1992b); (4) fastest development of

the nurse cell and collagen capsule (Pozio *et al.*, 1992b); and (5) very low resistance of larvae to freezing in muscles of all host species studied (Smith, 1975; Pozio *et al.*, 1992b; Malakauskas and Kapel, 2003). Although unpublished claims that *T. spiralis*, along with *T. britovi* and *T. pseudospiralis*, larvae can survive -18°C for four weeks in the muscles of ponies is provocative, these data require confirmation, because in another study, larvae of *T. spiralis* in a naturally infected horse did not survive freezing at -15°C for 24 hours (Pozio and Zarlenga, 2005).

Analysis of submissions to the ITRC indicated that *T. spiralis* was the species identified in 87% of all isolates from domestic pigs, 67% from wild boars (*Sus scrofa*), 88% from horses (*Equus caballus*), 79% from synanthropic rats and in the only two isolates from synanthropic armadillos (*Chaetophraactus villosus*) (ITRC). In many regions of the world this species has been transmitted to wildlife hosts (e.g. badgers, *Meles meles*; red foxes, *Vulpes vulpes*; wolves, *Canis lupus*; bears, *Ursus americanus* and *Ursus arctos*; mountain lions, *Felis concolor*; bobcats, *Lynx rufus*; raccoon dogs, *Nyctereutes procyonoides*) through exposure to garbage dumps or foraging near human settlements, where pork scraps and offal from slaughtered animals may be scattered in the environment (see Dick and Pozio, 2001). In many countries of the Americas (e.g. Argentina, Chile, the United States), Europe (e.g. France, Germany, Ireland, Lithuania, Poland, Spain) and Asia (e.g. Thailand), *T. spiralis* is a parasite of wildlife maintained in nature by a sylvatic cycle (Worley *et al.*, 1994; Pozio, 2000; 2001a; Pozio and Zarlenga, 2005; Rafter *et al.*, 2005).

T. spiralis is the etiological agent of most of the *Trichinella* infections in human beings and deaths around the world, and the pathology it causes, appears higher than that of other species, probably due to the higher number of newborn larvae produced by the females: the level of the inflammatory response in host tissues, especially muscle, does not seem to be directly related to invasion of the tissue by newborn larvae; in fact, a higher inflammatory response has been observed around *T. spiralis* larvae in the muscle tissue, compared to that produced by larvae of other encapsulated species irrespective of the worm burden (Bruschi F., personal communication). However, a high larval production equates to more severe clinical pictures (Pozio *et al.*, 1993; Bruschi *et al.*, 1999; Gomez Morales *et al.*, 2002).

3.1.2. *Trichinella nativa* Britov and Boev, 1972 (Genotype T2)

This species is usually characterized as the arctic or freeze-resistant species and is widespread among wildlife of the arctic and subarctic areas of the Holarctic region (i.e. Canada, Greenland, Alaska and New Hampshire in the United States, Byelorussia, Estonia, Finland, Latvia, Lithuania, Norway, Russia, Sweden, Siberia, China, Kazakhstan, Kyrgyzstan, and Tajikistan) (Figure 2). The southern distribution boundary has been tentatively identified between the isotherms -5°C and -4°C in January (Shaikenov and Boev, 1983; Shaikenov, 1992; Pozio *et al.*, 1998a; Pozio and La Rosa, 2000). The main biological features of *T. nativa* are (1) the length of the uterus is the shortest in comparison to that of *T. spiralis* and *T. nelsoni* (Sukhdeo and Meerovitch, 1977); (2) a low RCI in laboratory rodents (Rausch, 1970; Dick, 1983; Pozio *et al.*, 1992b) and in domestic and sylvatic swine (Rausch, 1970; Kapel and Gamble, 2000; Kapel, 2001); and (3) a high resistance to freezing in muscles of carnivores (up to 5 years) (Dick and Pozio, 2001); this biological character is lost, however, in frozen muscles of swine and rodents (Dick and Belosevic, 1978; Pozio *et al.*, 1992b; 1994a; Kapel, 2000; Malakauskas and Kapel, 2003).

The common hosts are terrestrial and marine carnivores living in arctic and subarctic areas (several species of mustelids; arctic fox; red fox; wolf; raccoon dog; domestic and sylvatic cats, *Felis silvestris*, *Felis eupilura*; lynx, *Lynx lynx*; Siberian tiger, *Panthera tigris*; black bear; brown bear; polar bear, *Ursus maritimus*; several species of seals; and walrus, *Odobenus rosmarus*). This species has rarely been detected in either domestic or wild swine (Pozio and Kapel, 1999). Since the advent of molecular methods for species confirmation, there have not been any documentations of natural infections of *T. nativa* in rodents or lagomorphs (Pozio, 2005); however, there are several reports of nematodes putatively identified as *Trichinella* sp. larvae in muscles of such wild hosts from arctic and subarctic regions (Rausch, 1970). The importance of sylvatic carnivores as reservoirs of *T. nativa* in nature is attested to by the finding that this parasite survives in these host's musculature for at least 20 years (Kumar *et al.*, 1990). *Trichinella nativa* is also the etiological agent of trichinellosis in human populations living in frigid zones, who acquire the infection from eating raw



Figure 2 World map showing the distribution areas of *Trichinella nativa* (Tna), *Trichinella britovi* (Tb), *Trichinella murrelli* (Tm), *Trichinella nelsoni* (Tne), *Trichinella* T6 (T6), *Trichinella* T8 (T8) and *Trichinella* T9 (T9). In some regions the distribution areas of these encapsulated species and genotypes overlap. (Redrawn from www.iss.it site Trichinella index.asp).

meat from reservoir hosts such as walruses, bears and other game animals (Rausch, 1970; Margolis *et al.*, 1979; MacLean *et al.*, 1989; Serhir *et al.*, 2001; Schellenberg *et al.*, 2003; Forbes, 2005; Moller *et al.*, 2005).

3.1.3. *Trichinella britovi* Pozio *et al.*, 1992 (Genotype T3)

Among sylvatic species, *T. britovi* has the widest geographical range, occurring in wildlife of the temperate areas of the Palearctic region, from the Iberian peninsula to the Far East (Pozio, 2000, 2001a) and extending southward to Northern and Western Africa (Nezri *et al.*, 2006; Pozio *et al.*, 2005b) (Figure 2). The northern geographic boundary appears to be determined by the isotherms -6°C to -5°C in January (Shaikenov and Boev, 1983; Shaikenov, 1992; Pozio *et al.*, 1998a; Pozio, 2000). In Palearctic regions, this species is sympatric with *T. nativa* between the isotherms -4°C and -6°C , and there are several reports of mixed infections in the same host from Estonia, Finland and Lithuania (Pozio *et al.*, 1998a; Pozio, 2000; Malakauskas, 2002; Oivanen *et al.*, 2002). This species is prevalent among sylvatic carnivores such as mustelids, viverridae (European genet, *Genetta genetta*; African palm civet, *Nandina binotata*; true civet, *Viverra civetta*), red foxes, jackals (*Canis aureus*), wolves and brown bears. In Europe, it has been identified in 83, 30 and 11% of isolates from red foxes, wild boars and domestic pigs, respectively (ITRC). This species was also detected in three horses that were the source of three outbreaks of human trichinellosis in Italy (Pozio and Zarlenga, 2005; International Commission on Trichinellosis, ICT). Infections in brown rats living in farms or garbage dumps has been reported in Italy and Estonia although larvae of this species have a very short survival time in this host (Pozio, 2000).

Certain important biological features of *T. britovi* can be considered intermediate between *T. spiralis* and *T. nativa*: (1) RCI in laboratory mice and rats and in domestic and sylvatic swine is lower than that of *T. spiralis*, but higher than that of *T. nativa* (Pozio *et al.*, 1992b; Kapel and Gamble, 2000; Kapel, 2001) and (2) larvae of *T. britovi* survive in frozen muscles of swine up to three weeks and up

to 11 months in fox muscle (Dick and Pozio, 2001), whereas the survival in frozen muscles of mice and rats ranged from three to seven days according to the isolate and the freezing temperature (Pozio *et al.*, 1992b, 1994a; Malakauskas and Kapel, 2003). This species can be transmitted to humans through the consumption of meat from wild boars, horses and domestic pigs (usually those raised in extensive grazing systems) and from sylvatic carnivores (e.g. red fox and jackal) (Pozio *et al.*, 2001d; Nezri *et al.*, 2006). The clinical picture is moderate or benign and no deaths have been documented to date, although this may be a consequence of infective dose size (Pozio *et al.*, 2003).

3.1.4. *Trichinella murrelli* Pozio and La Rosa, 2000 (Genotype T5)

This is a sibling species of *T. britovi*, apparently restricted to North America (Pozio and La Rosa, 2000). It occurs in sylvatic carnivores (e.g. bob cat; black bear; coyote, *Canis latrans*; raccoon; and red fox) and domestic animals (e.g. domestic dog, horse, cat) across the United States (California, Connecticut, Georgia, Illinois, Indiana, Maryland, New Mexico, Pennsylvania, Virginia and Texas) and in the Vancouver area of Canada (Minchella *et al.*, 1989; Snyder *et al.*, 1993; Yao *et al.*, 1997; Pozio and La Rosa, 2000; Pozio *et al.*, 2001e; Gajadhar *et al.*, 2004; ITRC) (Figure 2). The isotherm -6°C in January may be a determinant of its northern border of distribution. The southern limit is unknown due to the lack of adequate survey data from Mexico and Central America. A mixed infection of *T. murrelli* and *T. spiralis* larvae was detected in a black bear in California (ITRC). This species has not been detected as a natural infection in swine. The main biological features of this species are (1) very low resistance to freezing (Pozio *et al.*, 1992b, 1994a; Malakauskas and Kapel, 2003); (2) low infectivity for laboratory mice and rats (Pozio *et al.*, 1992b; Pozio and La Rosa, 2000), and domestic and sylvatic swine (Kapel and Gamble, 2000; Kapel, 2001), but high infectivity for *Peromyscus leucopus* and *Peromyscus maniculatus* (Minchella *et al.*, 1989; Yao *et al.*, 1997); and (3) very slow development of the collagen capsule around the larva in mouse muscles (Pozio *et al.*, 1992b). This

species has been identified in human outbreaks due to the consumption of meat from black bears in the United States (Roy *et al.*, 2003). A great deal of clinical information on this species was gained from a 1985 outbreak in France due to the consumption of horse meat imported from Connecticut (Ancelle *et al.*, 1988; Ancelle, 1998). The clinical picture is typically moderate or benign, probably due to low numbers of larvae in ingested meat meals, but severe cases leading to death have been reported (Ancelle, 1998; Ancelle *et al.*, 1988).

3.1.5. *Trichinella nelsoni* Britov and Boev, 1972 (*sensu* Pozio *et al.*, 1992) (*Genotype* T7)

The documented distribution of this species is restricted to eastern Africa, from Kenya to South Africa (Pozio *et al.*, 2005a), but this is based on only a few surveys and its range may be much broader (Figure 2). The host range includes Hyaenidae (spotted hyena, *Crocuta crocuta*, and striped hyena, *Hyaena hyaena*), Canidae (side-striped jackal, *Canis adustus*; black-backed jackal, *Canis mesomelas*; bat-eared fox, *Otocyon megalotis*; domestic dog), and Felidae (lion, *Panthera leo*; leopard, *Panthera pardus*; cheetah, *Acynonix jubatus*; and serval, *Felis serval*); it occurs at least occasionally in sylvatic suids (bush pig; wart-hog, *Phacochoerus aethiopicus*), some of which have been the source of human infections (Young and Kruger, 1967; Nelson, 1970; Sachs, 1970; Young and Whyte, 1975; Pozio *et al.*, 1994b; 1997b; ITRC). In over 1000 rodents in Africa, *Trichinella* sp. larvae were detected in only one (*Mastomys natalensis*) from the Kruger National Park of South Africa (Young and Kruger, 1967); however, since this Park is endemic for both *T. nelsoni* and *Trichinella* T8 (Pozio *et al.*, 2005b), the identity of the species cannot be assumed.

The main biological features of this species are (1) low infectivity to laboratory rodents (Nelson, 1970; Sukhdeo and Meerovitch, 1977; Pozio *et al.*, 1992b) and swine (Nelson, 1970; Kapel and Gamble, 2000; Kapel, 2001) compared to *T. spiralis*, but higher than that of *T. nativa*; (2) very low resistance to freezing in host muscles (Pozio *et al.*, 1992b; 1994a; Malakauskas and Kapel, 2003); and (3) the length of the uterus is intermediate between those of *T. spiralis* and *T. nativa* (Sukhdeo and Meerovitch, 1977).

Less than 100 human infections have been documented for this species in Kenya and Tanzania (Pozio *et al.*, 1994b). The clinical picture ranges from benign to severe and several deaths have been documented (Nelson, 1970; Bura and Willett, 1977); however, the pathology due *T. nelsoni* seems to be less than that for the other encapsulated species, because death was observed only in persons with more than 4000 larvae per gram of muscle, whereas those with 420–2800 larvae per gram recovered (Nelson, 1970; Bura and Willett, 1977). Other infections, including some deaths, attributed to *T. nelsoni* because of its geographic location and to the consumption of wild pigs, have been reported in Ethiopia (Perdomo Gonzalez *et al.*, 1986; Kefenie *et al.*, 1988; Kefenie and Bero, 1992; Gelnew, T., personal communication), although the species was never confirmed.

3.1.6. *Trichinella T6* Pozio *et al.*, 1992 (*Genotype T6*)

This North American genotype (Pozio *et al.*, 1992a) is widespread in carnivores (e.g. brown and black bears; wolves; gray fox, *Urocyon cinereoargenteus*; coyote; wolverine, *Gulo gulo*; fisher, *Martes pennanti*; mountain lion; bob cat). The distribution range is confined to the arctic and subarctic regions of the United States and Canada (Alaska, Idaho, Montana, Ohio, Pennsylvania, Wyoming and Ontario) (Worley *et al.*, 1990; Weyermann *et al.*, 1993; Pozio, 2000, 2001a; La Rosa *et al.*, 2003b; Gajadhar *et al.*, 2004) (Figure 2). This genotype is distinguished from *T. nativa* by biochemical and molecular characters, in spite of their ability to interbreed in both the laboratory (in two-way sex crosses) and naturally (hybrids have been found in wolves of Alaska) (La Rosa *et al.*, 1992, 2003b). The genetic differences between these two genotypes are probably due, at least in part, by geographical fragmentation of *T. nativa* colonization and subsequent evolutionary divergence during the glacial periods (La Rosa *et al.*, 2003b). Otherwise, *Trichinella T6* and *T. nativa* are very similar in biological features: (1) high freezing resistance of larvae in muscles of carnivores (Worley *et al.*, 1986; Dick and Pozio, 2001); (2) low infectivity to laboratory mice and rats (Pozio *et al.*, 1992b; Malakauskas and Kapel, 2003) and to domestic and sylvatic swine (Murrell *et al.*, 1985; Kapel and Gamble, 2000; Kapel,

2001). A few human infections have been documented, from the consumption of cougar and black bear meat in the United States (Idaho) and Canada (Ontario), respectively (Dworkin *et al.*, 1996; ITRC). The clinical picture is benign or moderate and no death has been documented, probably due to low infection dose, a typical circumstance in its hosts (low larval density).

3.1.7. *Trichinella* T8 Pozio *et al.*, 1992 (Genotype T8)

Trichinella T8 has been identified only from a lion of the Ethosa National Park of Namibia and a lion and a spotted hyena from the Kruger National Park of South Africa, where it lives in sympatry with *T. nelsoni* (Pozio *et al.*, 1992a, 1994b, 2005b) (Figure 2). This genotype can be easily distinguished by certain biochemical and molecular characters from *T. britovi* (La Rosa *et al.*, 1992, 2003a; Nagano *et al.*, 1999; Wu *et al.*, 1999; La Rosa and Pozio, 2000; Pozio *et al.*, 2005b) although they share similar biological characters and can interbreed in two-way sex crosses (Pozio *et al.*, 1992b; Pozio, E., unpublished data). The presence of this genotype in southern regions of Africa was proposed to be due to passive introduction from Europe during the European colonization, similar to that which has occurred for *T. spiralis* (La Rosa and Pozio, 2000). However, the recent finding of *T. britovi* in wildlife of West Africa suggests that *Trichinella* T8 is a geographic isolate (or subspecies) of *T. britovi*, which colonized Africa before *T. britovi* (Pozio *et al.*, 2005b). No human cases caused due to this genotype have been documented.

3.1.8. *Trichinella* T9 Nagano *et al.*, 1999 (Genotype T9)

Trichinella isolates originally identified as *T. britovi* from Japanese wildlife (raccoon dog; Japanese black bear, *Ursus thibetanus japonicus*) (Pozio *et al.*, 1996a), have now been shown by molecular methods to differ from the European strains, and are provisionally designated *Trichinella* T9 (Nagano *et al.*, 1999) (Figure 2). More recently, isolates from five red foxes of the Hokkaido Island, which were earlier erroneously identified as *T. nativa* (Yimam *et al.*, 2001), are now recognized as *Trichinella* T9 (ITRC). Although *Trichinella*

T9 and *T. britovi* interbreed under experimental conditions in two-way sex crosses, a recent analysis of the former's ITS2 sequence suggests that *Trichinella* T9 is genetically more closely related to *T. murrelli* than to *T. britovi* (Pozio and Zarlenga, 2005). No human cases caused due to this genotype have been documented.

3.2. The Non-Encapsulated Clade

Three species, infecting mammals and birds (one species) or reptiles (two species), compose this clade.

3.2.1. *Trichinella pseudospiralis* Garkavi, 1972 (Genotype T4)

For sometime after its discovery in 1972, *T. pseudospiralis* was considered an enigma (Dick, 1983), because only the initial isolate the "Garkavi's isolate" (Garkavi, 1972) existed. Also, its ability to infect birds appeared to be an anomaly (see Bessonov *et al.*, 1978). Nematode larvae resembling *Trichinella* sp. had been detected previously in muscles of some birds from Alaska, Iowa, California and Spain, but none could be confirmed as *Trichinella* (Rausch *et al.*, 1956; Zimmermann and Hubbard, 1969; Calero *et al.*, 1978; Wheeldon *et al.*, 1983). Eventually, new isolations of non-encapsulated larvae of *Trichinella* were made from birds (ravens, *Corvus frugilegus*) and mammals (corsac fox, *Vulpes corsac*; and Indian mole rat, *Bandicota bengalensis*) from Kazakhstan and India, and identified as *T. pseudospiralis* by breeding experiments using the Garkavi's isolate as a reference strain (Niphadkar, 1973; Shaikenov, 1980; Shaikenov and Boev, 1983). In the early 1990s, new foci of this parasite were discovered in Tasmania involving both marsupials and birds (Obendorf *et al.*, 1990; Obendorf and Clarke, 1992). Since then, this species has been reported in Asia, Europe and North America from domestic and sylvatic animals (Figure 3) (Shaikenov and Boev, 1983; Pozio *et al.*, 1992c; Lindsay *et al.*, 1995; Pozio *et al.*, 1999b, 2004b; Malakauskas, 2002; Oivanen *et al.*, 2002; van der Giessen *et al.*, 2004; Gamble *et al.*, 2005; Hurníková *et al.*, 2005; Nöckler *et al.*, 2006). In total, this species has been found in 14 mammalian species and seven



Figure 3 World map showing the distribution areas of the three populations of *Trichinella pseudospiralis* from Ne-arctic (TpsN), Palearctic (TpsP) and Australian (TpsA) regions. *Trichinella papuae* (Tpa) and *Trichinella zimbabwensis* (Tz). (Redrawn from www.iss.it/site/Trichinella/index.asp).

avian species (Pozio, 2005); the higher number of reports from mammals than from birds is likely the result of a greater number of examinations of mammals than birds. With regard to mammals, *T. pseudospiralis* of North America and Europe has been detected almost exclusively in wild boars and only twice in a lynx and in a red fox. The main biological features of *T. pseudospiralis* are (1) lack of a collagen capsule detectable by light microscopy around larvae in muscles (Xu *et al.*, 1997); (2) infective for both mammals and birds; (3) smaller size of newborn and muscle larvae and adults than that of all other *Trichinella* spp. and genotypes (Boev *et al.*, 1979; Dick, 1983; Lichtenfels *et al.*, 1983); (4) lower RCI in laboratory mice, rats and swine, both domestic and sylvatic, than that of *T. spiralis*, but higher than that of all other species and genotypes (Pozio *et al.*, 1992a; Kapel and Gamble, 2000; Kapel, 2001; La Rosa *et al.*, 2001); and (5) low resistance to freezing (Pozio *et al.*, 1992b; Malakauskas and Kapel, 2003). The RCI in birds is lower than that in rodents (La Rosa *et al.*, 2001); however, this difference may be due to selection pressure by the constant maintenance in laboratory mice for many years.

Three *T. pseudospiralis* geographical populations from the Palearctic, Nearctic and Australian (Tasmania) regions can be distinguished by molecular markers in the expansion segment five (ES5) (Zarlenga *et al.*, 1996; La Rosa *et al.*, 2001). At this locus, individual larvae from the Tasmanian isolate exhibits three distinct banding patterns characterized by either one band of 310 bp, two bands of 310 and 320 bp or 310 and 340 bp; individual larvae from Nearctic isolates exhibit two bands of 300 and 310 bp; whereas, larvae from the Palearctic show two bands of 290 and 300 bp (La Rosa *et al.*, 2001). Biochemical analysis of 12 allozymes also reveals different patterns at the PGM locus between the Palearctic isolates and those from the Nearctic and Australian regions (La Rosa *et al.*, 2001). In addition, a biochemical polymorphism has been detected at the PGM locus between two Palearctic isolates originating from Caucasus (the Garkavi's strain) and one isolate from a domestic pig of the Slovak Republic (Hurníková *et al.*, 2005).

A single human case, probably acquired in Tasmania, and three outbreaks involving 92 people, in Kamchatka, Thailand and France have been documented (Andrews *et al.*, 1995; Britov, 1997; Jongwutiwes

et al., 1998; Ranque *et al.*, 2000). Infections in people range from clinically moderate to severe, with one death (Jongwutiwes *et al.*, 1998).

3.2.2. *Trichinella papuae* Pozio *et al.*, 1999 (Genotype T10)

Non-encapsulated larvae of *Trichinella* sp. were discovered in 1988 in the muscles of domestic sows and wild pigs (hybrids between *Sus scrofa* and *Sus celebensis*) of south-west Papua New Guinea (PNG), near the border with Irian Jaya (Owen *et al.*, 2000). Biological and molecular studies demonstrated that these parasites were a new species, subsequently named as *T. papuae* (Pozio *et al.*, 1999a). This species has now been detected in farmed saltwater crocodiles (*Crocodylus porosus*) of PNG (Pozio *et al.*, 2005a) (Figure 3). In experimental infections, this species exhibits a high RCI in caimans and monitor lizards, but a very low RCI in turtles and pythons (Pozio *et al.*, 2004a); its RCI in red foxes is similar or higher than that of *T. spiralis* (Webster *et al.*, 2002). The main biological features are (1) lack of a collagen capsule detectable by light microscopy around the muscle larvae; (2) infectivity for both mammals and reptiles, but not birds; (3) lower RCI in laboratory mice and rats than that of *T. spiralis*, *T. britovi* and *T. pseudospiralis*, but higher than that of the other encapsulated species and genotypes (Pozio *et al.*, 1999a); (4) muscle larva and adult sizes similar to that of encapsulated species and genotypes, but greater than that of *T. pseudospiralis* (Pozio *et al.*, 1999a); and (5) low resistance to freezing (Webster *et al.*, 2002).

Based on the ES5 sequence, two distinguishable populations have been identified so far in PNG (Pozio *et al.*, 2005a). Although this parasite has never been isolated from humans, a high percentage of people living in regions where only this *Trichinella* spp. appears to exist among wild pigs have specific antibodies against *Trichinella* antigens (Owen *et al.*, 2001, 2005). This discovery of a *Trichinella* spp. infecting both mammals and reptiles may provide an explanation for earlier reports of human outbreaks attributed to the consumption of turtle and brown lizard meat in Thailand (Khamboonruang, 1991).

3.2.3. *Trichinella zimbabwensis* Pozio *et al.*, 2002 (Genotype T11)

This species is very similar to *T. papuae* with which it shares important biological features. However, *T. zimbabwensis* is distinguished from *T. papuae* by three diagnostic allozymes (GLD, MPI, PGM), differences in the ES5 sequence (88% similarity), the cytochrome oxidase I (91% similarity), and mt-lsrDNA (96% similarity) (Pozio *et al.*, 2002).

This recently described species has been detected only in reptiles of Africa, although experimentally it is able to infect mice, rats, hamsters, foxes, pigs and monkeys (Mukaratirwa and Fogin, 1999; Mukaratirwa *et al.*, 2001, 2003, 2005; Pozio *et al.*, 2002; Hurníková *et al.*, 2004). When first discovered in 1995, *T. zimbabwensis* larvae were detected in 256 (39.5%) farmed Nile crocodiles (*Crocodylus niloticus*) from 18 (62.1%) Zimbabwe crocodile farms. This parasite has also been detected in sylvatic monitor lizards (*Varanus niloticus*) from two localities in Zimbabwe, in sylvatic Nile crocodiles from Lake Cahora Bassa in Mozambique and in a farmed Nile crocodile from Lake Abaja in Ethiopia (Pozio and Zarlenga, 2005) (Figure 3). Human infections are yet to be reported.

4. PHYLOGENY

The current taxonomic scheme for the genus *Trichinella* originated from biochemical studies (allozymes) on 152 *Trichinella* isolates belonging to eight taxa (Pozio *et al.*, 1992a) and led to the creation of the first extensive dendrograms (La Rosa *et al.*, 1992). These were later corroborated by Bandi *et al.* (1995) who used RAPDs and allozymes to independently generate congruent trees. In 1997, Zarlenga used mitochondrial DNA data to produce a distance-based (UPGMA) tree that strongly supported the topology of Bandi *et al.* (1995). Independent trees were later generated from all 11 recognized species and genotypes of *Trichinella* by neighbour joining (NJ) and UPGMA using multilocus enzyme electrophoresis data (La Rosa *et al.*, 2003a). Although the latter trees of La Rosa *et al.* (2003a) showed remarkable similarity with each other, they raised questions regarding the overall topology relative to

those of Bandi *et al.* (1995) and Zarlenga (1997) because they did not completely delineate non-encapsulated species as a monophyletic clade, but placed them at the base of the *Trichinella* tree (Pozio and Zarlenga, 2005). In 2004, Gasser *et al.* sequenced the D3 domain of the nuclear ribosomal DNA from all currently recognized species and genotypes of *Trichinella* and demonstrated strong bootstrap support for monophyly among *T. spiralis* and *T. nelsoni*, and among *T. nativa* and *Trichinella* T6 using maximum likelihood, parsimony and or NJ methods. In this analysis, the non-encapsulated species clustered at the base of the tree and isolates of the bird-related species (*T. pseudospiralis*) clustered independent of those species related to reptiles (*T. papuae* and *T. zimbabwensis*).

In the recent past, scientists believed that extant, non-encapsulated species of *Trichinella* coevolved first with lower vertebrate classes (e.g. reptiles and birds), and then later in mammals, followed by the evolution of the encapsulated species, which was restricted to mammals only (Pozio *et al.*, 2002, 2004a). More recently, Zarlenga *et al.* (2006) analyzed the phylogeny of *Trichinella* using the variation in three genes (nuclear SSU rDNA and ITS2; mitochondrial LSU rDNA and COI DNA) from all 11 recognized species and genotypes. The results showed that the extant species of *Trichinella* probably diversified only within the last 10–20 million years and coincided with the divergence of Suidae from the Tayassuidae (Bowen *et al.*, 2002) in the Lower Miocene (Figure 4). In addition, *T. spiralis*, which anecdotally has been considered a crown species due to its strong association with domestic pigs, synanthropic rats and humans (Britov, 1982), appears in the most recent analysis to be basal to the encapsulated clade where the timeframe for divergence is constrained to the Lower Miocene (Zarlenga *et al.*, 2006).

5. BIOGEOGRAPHY

The biogeographic history of the encapsulated species and genotypes of *Trichinella* has recently been thoroughly investigated for the first time by Zarlenga *et al.* (2006). Since *T. spiralis* is the basal species of the encapsulated clade and exhibits high genetic variability among localized populations in Eastern Asia (La Rosa, unpublished data),

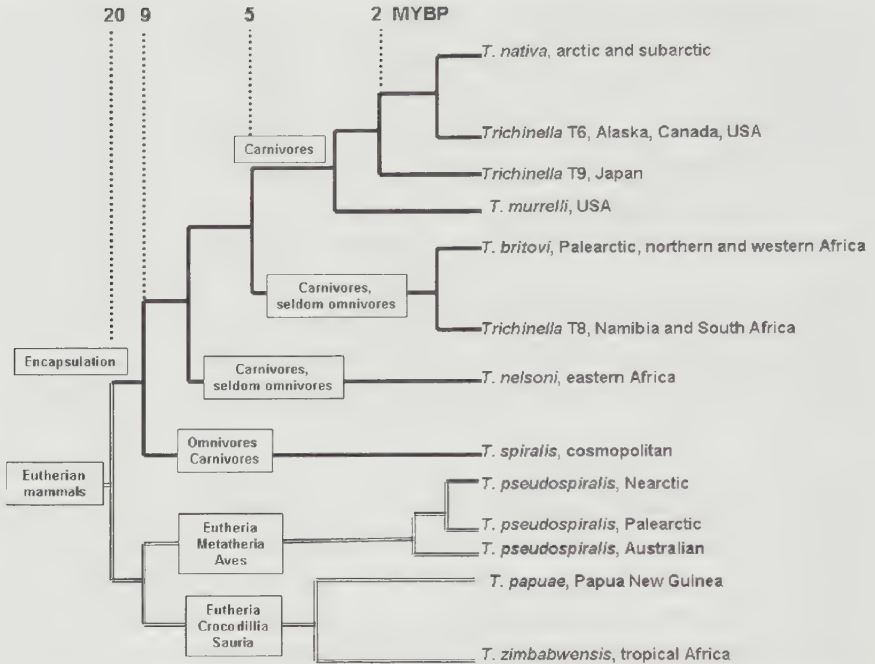


Figure 4 Phylogeny for species of *Trichinella* showing host associations and primary traits for life history reconstructed from variation in mtLSU and COX I mitochondrial genes and redrawn from Zarlenga *et al.* (2006). Bold tree = encapsulated clade infecting only mammals; Parallel line tree = non-encapsulated clade infecting both mammals and birds (*T. pseudospiralis*) or both mammals and reptiles (*T. papuae* and *T. zimbabwensis*). MYBP = million years before present.

these authors identify this geographical region as the probable origin of the encapsulated clade. In prior phenetic (Bandi *et al.*, 1985; Zarlenga, 1997; La Rosa *et al.*, 2003a) and phylogenetic (Gasser *et al.*, 2004) trees, the placement of *T. nelsoni* has always occurred at or near the base of the encapsulated clade. A tree placing *T. nelsoni* basal to *T. spiralis* would support an “out-of-Africa” event in which *T. nelsoni* could have expanded into the Palearctic, and the introduction of *Trichinella* T8 and *T. britovi* into Africa would have occurred later. However, the data and tree put forth by Zarlenga *et al.* (2006) suggests that the occurrence of *Trichinella* T8 and *T. britovi*, as well as *T. nelsoni*, in Africa (Pozio *et al.*, 2005b) is likely the result of

three independent expansion events from Eurasia following the land connections that formed during upper Miocene and into the Pleistocene. The expansion of *T. britovi* into northern and western Africa is likely the most recent event given the biochemical congruence among these isolates and those from Western Europe (Pozio *et al.*, 2005b).

According to Zarlenga *et al.* (2006), ursids, canids and felids are principally responsible for the radiation of Holarctic species throughout Europe and into North America through Beringia where vicariance speciation during the Quaternary was likely the driving force for divergence among many of the crown species. The unclassified status for *Trichinella* T6 suggests that evolutionary forces driving the speciation of the freeze-resistant genotypes are both recent and unresolved; however, La Rosa *et al.* (2003b) have identified natural hybrids of these two genotypes in Alaskan wolves. The divergence of these two genotypes may have resulted from environmental factors causing a bifurcation of the freeze-resistant genotype (*T. nativa* and *Trichinella* T6). The limited amount of information on non-encapsulated species, due in part to their recent discovery, the lack of sufficient numbers of isolates, and the worldwide dissemination of *T. pseudospiralis*, presumably resulting from migratory avian hosts, does not permit a deep understanding of the biogeography of this clade at this time.

There are many instances of sympatry among the *Trichinella* spp. Some examples are *T. nativa* (and the closely related genotype *Trichinella* T6) and *T. murrelli* in the United States and Canada (Pozio and La Rosa, 2000; La Rosa *et al.*, 2003b); *T. nativa* and *T. britovi* in Europe and Asia (Shaikenov and Boev, 1983; Shaikenov, 1992; Pozio *et al.*, 1998a); and *T. nelsoni* and *Trichinella* T8 in South Africa (Pozio *et al.*, 2005b) (Figure 2).

The distribution area of *T. spiralis* (Figure 1), which has been passively disseminated by humans and their domestic and synanthropic animals, also overlaps with that of *T. nativa*, *T. britovi* and *T. murrelli* in many regions (Figure 2) (Shaikenov and Boev, 1983; Pozio, 1998; Pozio and La Rosa, 2000). The non-encapsulated species *T. pseudospiralis* (Figure 3) has been detected in the same regions where *T. spiralis*, *T. nativa*, *T. britovi* and *T. murrelli* occur. In some regions of Africa, the distribution area of the non-encapsulated species *T. zimbabwensis* may

overlap with those of *T. britovi*, *T. nelsoni* and *Trichinella* T8, but additional data is needed to confirm this. The overlapping of these distribution areas has occasionally resulted in mixed infections in natural hosts (*T. spiralis* with *T. nativa*, or *T. britovi*, or *T. murrelli*, or *T. pseudospiralis*; *T. nativa* with *T. britovi* or *Trichinella* T6; and *T. britovi* with *T. pseudospiralis*) (Pozio *et al.*, 1997a, 1998a; Malakauskas, 2002; Oivanen *et al.*, 2002; La Rosa *et al.*, 2003b; Nöckler *et al.*, 2006; ITRC).

The discovery of mixed species infections in some regions provides some insight into the frequency of infection of sylvatic hosts. Although in experimental conditions many hosts develop an acquired immunity to secondary infection, such immunity may not be strong enough to prevent reinfection under natural conditions. For example, immunity depends to a large degree on the dose (infection level); therefore, strong protection from a natural initial infection may not always result because the larval density in wild animals is usually quite low (Kazura, 1982; Wakelin and Denham, 1983; Murrell 1985; Marti and Murrell, 1986). Further, in frequent transmission situations, re-infection may occur before development of protective immunity from a primary infection. Multiple species infections suggest, then, that high levels of exposure exist in some circumstances. Repeated infections could also promote the gene flow between larvae belonging to the same species, even if this flow cannot be directly demonstrated because of adult worm mating between rapidly acquired infections (Zarlenga, 1994; Pozio *et al.*, 1997a). Another factor important in the development of any immune barrier to repeated infections is the nutritional status of the host, which in some seasons can be less than optimal due to restrictions in food availability. Nutritional component levels for protein, selenium and vitamin A are strongly linked to host cellular immunity and resistance to micro- and macro-parasites (Solomons and Scott, 1994; Pedersen and Murrell, 2001; Pedersen *et al.*, 2002).

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Although *T. spiralis* was first discovered in domestic animals (see historical review by Campbell, 1983a), all other species of this genus

are primarily parasites of wildlife. The importance of wildlife as reservoir hosts for all species of *Trichinella* is underscored by the parasite's biomass, which is greater in wild than in domestic animals, unlike other nematode infections involving both sylvatic and domestic animals. When humans fail in the proper management of domestic animals and wildlife, *Trichinella* sp. (especially *T. spiralis*) infection is transmitted from the sylvatic environment to the domestic one, sometimes through synanthropic (intermediary between domestic and sylvatic) animals. In addition, some species can transfer in a reversible path from domestic animals to wildlife.

6.1. The Sylvatic Cycle

The sylvatic cycle occurs in all continents with the exception of Antarctica, where there is neither a record of this nematode nor evidence of any searches for it, especially in marine mammals. Differences in species cycles exist because of differences in reservoir host species and between regions, especially in areas where two or more species are present (Figure 2).

6.1.1. Natural Hosts

Although natural *Trichinella* infections have been reported in more than 100 species of mammals belonging to 11 orders (Marsupialia, Insectivora, Edentata, Primates, Lagomorpha, Rodentia, Cetacea, Carnivora, Perissodactyla, Artiodactyla and Tylopoda), those from Insectivora, Lagomorpha, Cetacea, Tylopoda and infections in most of Rodentia, are problematic and need confirmation (Pozio, 2005). In experimental infections, these parasites are able to complete their life cycle in all species of mammals tested, but only a few of these appear to play an important role in the sylvatic and or domestic cycle.

The transmission cycles of the different sylvatic species and genotypes are closely related to their host species ecologies. For example, in Europe, *T. spiralis* and *T. britovi* occur almost equally in wild boars (49% and 47%, respectively), with some differences related to the habitat characteristic and human behavior at the country level, whereas the same two species occur with quite different frequencies in red foxes and

other sylvatic carnivores (7% and 92%, respectively) (ITRC). A similar pattern occurs in North America, where *T. spiralis* and the sylvatic species *T. murrelli*, and *T. nativa*, and the genotype *Trichinella* T6, have been detected in 12% and in 87% of sylvatic carnivores, respectively (ITRC). These prevalence figures, based on data from over a thousand examinations, are not completely in agreement with data from experimental infections, which do not reveal great differences in the host susceptibilities (Kapel, 2000). Data from experimental infection with laboratory animals is not always in agreement with findings from natural populations. As a rule, wild animals encounter over time many pathogens (including helminths), which potentially could influence the immune response to *Trichinella* sp. Unlike the situation with wild animals, infections of clean, naïve laboratory animals which have very different encounters with other infectious agents and normally are on a high level nutritional plane could react quite differently than the same species reared under natural conditions. Further, the genetics of the host may have a dramatic effect on its immune response, especially when comparing rodents to animals such as swine (Murrell *et al.*, 1987b). In addition, the biology of the different *Trichinella* spp. in host carrion cannot be easily evaluated in laboratory animals raised and maintained under controlled conditions, shielded from the influence of the natural habitat. Therefore, we believe that experimental data from laboratory animals, should be interpreted with caution.

The host range for the sylvatic cycle is determined by the potential host species available in the different regions (Pozio, 2000, 2001a). Because swine are not a suitable host for *T. nativa*, *T. murrelli* and *Trichinella* T6 (Kapel and Gamble, 2000; Kapel, 2001), these animals do not play a role as a reservoir for these pathogens in the regions of Eurasia and North America, although there is an occasional report of *T. nativa* in wild boars (Pozio and Kapel, 1999).

Among primates, only humans have been naturally infected with *Trichinella* spp., although experimental infections in monkeys with *T. spiralis* or *T. pseudospiralis* have demonstrated the high susceptibility of these hosts (McCoy, 1932; Kocieka *et al.*, 1981); the ecological consequences of these infections are severe for the parasite since in the absence of cannibalism, they normally represent a dead end. Evidence suggests that the infection of horses (*Perissodactyla*), rodents

(especially rats) and edentata (armadillos) occurs most commonly where poor livestock rearing practices (exposure to infected meat) exists. The reservoir role of Marsupialia is also limited, it has only been documented in Tasmania (Obendorf *et al.*, 1990), and in in opossums (*Didelphis* spp.) in North America, where the parasite is widespread among placental mammals; however, its role as a reservoir is unknown (Solomon and Warner, 1969; Zimmermann 1970; Schad *et al.*, 1984; Murrell *et al.*, 1985; Leiby *et al.*, 1988). In spite of the potential broad host spectrum of *Trichinella* spp., the greatest biomass of these parasites occurs among the Carnivora (Campbell, 1983b; Pozio *et al.*, 1997b, 2005b; Pozio and Dick, 2001) and the artiodactylid family Suidae (mainly domestic pigs, different races of wild pigs, wild boars, bush pigs and warthogs) (Nelson, 1970; Sachs 1970; Campbell, 1983b; Pozio *et al.*, 1999a, 2005a; Pozio, 2005). Natural infections in other artiodactylid species, both sylvatic (reindeer and roe deer), and domestic (sheep, goat and cow) are sporadic (Murrell, 1994; Takahashi *et al.*, 2000; Pozio, 2001a).

Seven species of birds are documented as hosts for *T. pseudospiralis*, and six other species suspected, but unconfirmed; these hosts belong to the orders Strigiformes (four species), Ciconiiformes (eight species) and Passeriformes (one species) (Shaikenov, 1980; Pozio *et al.*, 1992c; Lindsay *et al.*, 1995; Pozio, 2005; Garkavi, B.L., personal communication). The role of birds as reservoirs of this species needs evaluation; however, the low inter-regional genetic variability and the discontinuous distribution of *T. pseudospiralis* in the Nearctic and Palearctic regions could be explained by the transmission through birds because of the latter's mobility and wide distribution patterns (La Rosa *et al.*, 2001). The recentness of the discovery of *T. papuae* and *T. zimbabweensis* infections in certain reptile species (Pozio *et al.*, 2004a, 2005a) and because so few surveys for them have been conducted, it is impossible to speculate on the role of other vertebrates in the ecology of these species.

6.1.2. *The Parasite's Adaptation to the Environment*

An important adaptation of the parasite, which facilitates its transmission, is the physiological mechanism utilized by muscle larvae to

promote its survival in decaying carcasses; the greater the persistence of larval viability, the higher the probability of being ingested by a scavenging host. In spite of the larva-induced angiogenic process that develops around the nurse cell after larval penetration of the muscle cell, larval metabolism is basically anaerobic (Despommier, 1990), which favors its survival in decaying tissues, probably longer for the encapsulated than for the non-encapsulated species (Stewart *et al.*, 1990). The persistence of larvae in putrefying flesh is, of course, also determined by the environment: high humidity and low temperatures favor survival even when the muscle tissue is completely liquefied. This condition has been proposed as the environment of the "free-living" stage, resembling the egg stage of most of other nematode species (Madsen, 1974).

The importance of this stage in the natural cycle of the parasite is underscored by the survival of muscle larvae of species in frozen muscles of carrion for one (*T. britovi*) or more years (*T. nativa* and *Trichinella* T6) (Dick and Pozio, 2001). The anaerobic metabolism favoring the survival in putrefying flesh, along with the ability of larvae of some species to survive freezing, are two separate mechanisms that strongly increase the survival of the parasite in nature. Survival is greatest at temperatures between 0 °C and -18 °C. At lower temperatures, survival time is reduced, suggesting that the optimal temperature range for survival to freezing corresponds to the temperature under the snow.

6.1.3. *The Human Influence*

The sylvatic cycle may also be influenced by human actions. For example, the common habit of hunters to leave animal carcasses in the field after skinning, or removing and discarding the entrails, increases the probability of transmission to new hosts (Cironeanu, 1974; Madsen, 1974; Batkaev and Vakker, 1992; Worley *et al.*, 1994; Pérez-Martin *et al.*, 2000; Pozio *et al.*, 2001f).

Epidemiological surveys carried out in Europe, North America and Africa have shown that *Trichinella* spp. are more prevalent in wild animals living in natural or undisturbed areas such as parks and

forests (Worley *et al.*, 1994; Pozio *et al.*, 1997b), protected areas and mountain regions (Pozio *et al.*, 1996b, 2005b; Pozio, 1998) where the human activity has not strongly changed the habitat, even when suitable hosts are present in areas where human activity is strong. A possible explanation is that in undisturbed areas of these three continents, hosts of *Trichinella* spp. which have predominantly scavenger and cannibalistic behaviors, practice their natural trophism, whereas in habitats where human activity is high, the number of potential hosts is reduced or even if not, the animals have access to alternative food resources resulting from human activity (e.g. synanthropic and domestic animals, garbage). This is the case of several European countries, where the sylvatic cycle is nearly absent in areas characterized by a strong human influence, but present in mountain and/or protected areas (Pozio *et al.*, 1996b; Pozio, 1998).

Since the prevalence of *Trichinella* infection increases with the host age, a question needing study is how the age structure of potential host populations affects transmission. This is important because in natural areas where the human impact is low or absent, animal populations generally have a wide age range, but in areas influenced by the human activity, these potential host populations tend to be skewed toward young individuals, most likely immigrants in search of a new home range (MacDonald, 1980).

Human-caused perturbations of the sylvatic environment may also affect the epidemiological patterns of human and animal trichinellosis, a phenomenon well documented for many animal pathogens (Daszak *et al.*, 2000). A case in point is the consequences from the increase in forests and fallow land, concomitant with a decrease in farms, in Europe over the past 100 years, which has facilitated a great enlargement in the regions of wild boar populations and increased transmission of *Trichinella* spp. to animals and humans (Pozio *et al.*, 1996b). In France, there was a nine-fold increase between 1975 and 2000 in wild boars, which is of concern to outdoor farming because of the risk of transmission of *Trichinella* spp. from wild boars to domesticated pigs (Dupouy-Camet, 2000). This risk should be of concern to public health authorities in the United States, where the wild pig populations (feral pigs, Eurasian wild boars and their hybrids) have grown dramatically since the recent decades, and now have

extended their range to 31 states and number between 4 and 5 million (Mayer and Brisbin, 1991; Frazier, 2005). Along with considerable environmental damage, the risk of transmission of *Trichinella* spp. to other game animals and outdoor pig farms has increased. Along with a potential effect on safety of domestic pig consumption, an impact on export of meat could occur, as happened in France in 1998, when vacuum-packed meat sold as wild boar imported from the United States caused an outbreak of trichinellosis in humans in Normandy (Dupouy-Camet, 2000), a food safety issue not unlike that for imported horse meat (see Section 6.2.2).

6.1.4. *The Role of Micromammals*

There is a lack of consensus among scientists on the role of micromammals (mainly rodents and insectivores) in the sylvatic cycle due to the usually low number of infections in their populations. Most records on infection in these hosts date back before *Trichinella* isolates could be confirmed and typed by biological, biochemical or molecular methods; therefore, the role these animals play is uncertain. Identifications in some reports of nematode larvae from these hosts, on purely morphological grounds, as *Trichinella* sp. needs verification and should probably be considered as rare events (Merkushev, 1970; Rausch, 1970; Zimmermann, 1971; Holliman and Meade, 1980; Bessonov, 1981; Pozio, 2005). Among the non-*Trichinella* nematode larvae that can be detected in muscles, are those from the gut, which may contaminate muscles during necroscopy. Only skilled and experienced microscopists can identify these larvae correctly.

Because the number of micromammals living on the home range of a single carnivore is, as a general rule, a thousand-fold greater than for carnivores, prevalence studies for *Trichinella* is difficult, given that worm burdens in sylvatic carnivores and omnivores is generally low (e.g. 1.0 larvae/g or less, in preferential muscles). Therefore, the chance that a micromammal of 10–20 g of body weight will ingest enough flesh of infected carrion to permit transmission and development of at least one male and one female larva is very low. Another issue is that 90% of micromammals live less than 10 months (Burton and Pearson, 1987),

reducing their role in transmission compared to longer-lived carnivores. This question on the role of micromammals can best be answered by large investigations of micromammals in areas where there is a relatively high prevalence of *Trichinella* sp. in carnivores.

6.1.5. *Trichinella* spp. in Lower Vertebrates and in Invertebrates

There is a single report of experimental infections of amphibians (frogs and axolotls) with *T. spiralis*, in which it was observed that the development of larvae in the muscles was incomplete (Gaugusch, 1950). Attempts to infect fish with either *T. spiralis*, *T. britovi*, *T. pseudospiralis*, *T. papuae* or *T. zimbabwensis* have also failed (Guevara Pozo and Contreras-Pena, 1966; Tomasovicova, 1981; Moretti *et al.*, 1997; Pozio and La Rosa, 2005).

In addition to vertebrates, there are older reports describing the identification of encapsulated larvae of *Trichinella* in the intestine of adult insects and fly maggots (*Musca domestica*) and their potential role as paratenic hosts for *T. spiralis* (Merkushev, 1955). Several species of insects were experimentally infected and infective, non-encapsulated larvae of *T. spiralis* were observed up to eight days p.i. (Merkushev, 1955). More recently, infective *T. spiralis* larvae were detected in maggots of *Sarcophaga argyrostoma* experimentally fed on *T. spiralis*-infected mouse carcasses after up to five days p.i. at 8 °C and for shorter periods of time at higher temperatures (Maroli and Pozio, 2000).

Infection of a dog by feeding it 5000 amphipods that had been fed infected bear meat was reported by Fay (1968). Others have shown that seven species of crustaceans (amphipods and shrimp), trapped in the Arctic sea and fed on rat muscles infected with *T. spiralis* larvae, can retain larvae only up to 28 hours after infection in cold seawater (Hulebak, 1980). These results suggest that invertebrates play a very limited role, if any, in the dissemination of *Trichinella* sp. larvae in nature.

6.2. The Domestic Cycle

This cycle occurs where there are high-risk farming practices such as the intentional feeding of food waste, potentially containing pork

scraps (Gamble *et al.*, 2000), or unintentionally through exposure to carcasses of dead swine (Hanbury *et al.*, 1986), or infected wildlife, usually by unsecured free-range pasturing (Murrell *et al.*, 1987a; Pozio, 2000). A comprehensive picture of the domestic cycle also includes certain other transmission sources: (1) pigs allowed to scavenge on garbage dumps (Campbell, 1983b); (2) feeding of wild game carcasses or scraps from hunting (Pozio *et al.*, 2001f); (3) horses fed with pork scraps or with carcasses of fur animals (Pozio *et al.*, 2001a; Murrell *et al.*, 2004); (4) sled dogs fed with carcasses of other dogs or of game in the Arctic (Madsen, 1974); (5) the use of carcasses of slaughtered fur animals as food for other fur animals present at the farm (Madsen, 1974; Miller *et al.*, 2006); (6) the use of meat of slaughtered crocodiles to feed other farmed crocodiles as observed in Zimbabwe (Pozio *et al.*, 2002); and (7) the use of pork scraps to feed young crocodiles as recently demonstrated in Papua New Guinea (Pozio *et al.*, 2005a).

The most common etiological agent of the domestic cycle is *T. spiralis*, which is very well adapted to swine and in which it exhibits a very high reproductive rate without inducing serious pathology except in very high level of infection (Gould, 1970; Kapel and Gamble, 2000; Kapel, 2001). Occasionally, *T. britovi* can be transmitted in the domestic cycle, when humans feed pigs with game meat scraps or "pasture" pigs in refuse dumps containing carcasses of sylvatic animals (Pozio *et al.*, 2001d). *T. pseudospiralis* has been also transmitted to domestic pigs and rats on farms in Kamchatka, Russia and Slovak Republic (Pozio, 2001a; Hurníková *et al.*, 2005). *Trichinella*-infected meat scraps or carcasses of domestic animals from villages, farms and garbage dumps can be the source of infection for both synanthropic and wild animals circulating in the local habitat favoring the increase of the parasite biomass in the sylvatic cycle (spillover).

6.2.1. The Role of Rats

In the domestic habitat, where *Trichinella* is circulating among domestic animals, the brown rat is frequently found to be infected with *T. spiralis* and infrequently with *T. britovi* (Pozio *et al.*, 1996b) or

T. pseudospiralis (Britov, 1997; Oivanen *et al.*, 2002; Hurniková *et al.*, 2005). The role of this animal in the epidemiology of *Trichinella* continues to be debated as to whether it is a true reservoir host (sustaining the infection in the habitat in the absence of introductions of the parasite by other host species) or functions primarily as a vector of *Trichinella* (because of accidental infection) to domestic hosts (Haydon *et al.*, 2002). In the 19th century, Leuckart proposed a "Rat Theory", which implicated rats as a major reservoir of *T. spiralis* infection for domestic pigs. Zenker (1871) on the other hand, suggested that the infection in rats was merely an indicator of *Trichinella* sp. exposure risk in the area and that the real source of infection for both pigs and rats was meat scraps and offal of infected pig carcasses.

Although *T. spiralis* infection in pigs is often associated with infection in rats living in abattoirs (Schenone *et al.*, 1994; Bianli *et al.*, 2001), farms (Smith and Kay, 1987; Leiby *et al.*, 1990; Stojcevic *et al.*, 2004) and garbage dumps (Robinson and Olsen, 1960; Zimmermann and Hubbard, 1969; Mikkonen *et al.*, 2005), the results from various investigations are not conclusive (Schad *et al.*, 1987; Stojcevic *et al.*, 2004). There are no reports showing *T. spiralis* infection in brown rats where pig populations unequivocally have been found to be negative or in farms where pigs do not exist (Gomez Villafane *et al.*, 2004), although Mikkonen *et al.* (2005) propose that the continuing presence of *T. spiralis* in dump rats in Finland is facilitated by rat-to-rat transmission through cannibalism. Regardless of their capability to act as a true reservoir, there is substantial evidence that they can play a role in transmitting *T. spiralis* to pigs and must be considered in any design of an on-farm control program (Schad *et al.*, 1987; Gamble *et al.*, 2000; Oivanen *et al.*, 2002). Rat-control campaigns and farm renovations, however, must be done with care and incorporate an area-wide approach, because while these actions may solve the local problem, it could force infected rats to migrate and spread the infection to neighboring farms and villages. Evidence for this was reported by Smith *et al.* (1976) in some swineherds of the Atlantic provinces of Canada. In addition, the use of rat pesticides can actually favor the transmission, because poisoned rats are easy prey for pigs. Their role of vector can be amplified if pigs are not adequately fed, forcing these animals to eat

rats. This is consistent with findings in the United States that the occurrence of *T. spiralis* infection in domestic pigs greatly decreased when feeding with uncooked garbage and offal was terminated, which was implemented to control bacterial and viral infections (Hall, 1937).

6.2.2. *Trichinella Infection in Horses*

The emergence of equine trichinellosis is an intriguing story with still puzzling and unresolved aspects. As early as the late 19th century, there have been reports of both experimental infections with *Trichinella* sp. larvae in horses in Germany and Austria (Gerlach, 1873; Csokor, 1884) and a natural infection in Ohio (Thornbury, 1897); however, the potential role of horses in the transmission to humans was largely ignored until 1975, when an outbreak of trichinellosis occurred among 89 persons in Italy, who had eaten horse meat (Mantovani *et al.*, 1980). In the same year, another outbreak occurred in France (Bouree *et al.*, 1979), prompting the European Union to examine thousands of horses for the presence of *Trichinella* sp. larvae, adopting the method used to detect this infection in pigs (i.e. artificial digestion of 1.0 g of diaphragm pillar muscle) (Pozio *et al.*, 2001a). No natural infection in horses was detected at that time. However, between 1975 and 2005, human outbreaks of trichinellosis have occurred in France (2296 persons in eight outbreaks) and Italy (1038 persons in seven outbreaks), from the consumption of meat from individual horses imported from Canada, the former Yugoslavia, Mexico, Poland and the United States (Pozio and Zarlenga, 2005; ITRC). Routine examinations at the slaughterhouse had revealed infection in only one of the 15 horses involved in these outbreaks; unfortunately, the meat from even that horse was erroneously placed on the market (Pozio *et al.*, 1998b). The failure to detect infection in the other 14 horses was probably due to the examination of an inadequate quantity of muscle tissue (i.e. 1.0 g). Since then, the requirement for 5–100 g for testing was instituted, and *Trichinella* sp. larvae have been detected in a total of 18 horses bred in the former Yugoslavia, Mexico, Poland, Romania and Serbia (Pozio and Zarlenga, 2005). Worldwide, the prevalence of horse infection appears to be very low, with only 32 infections reported since 1975

(horses that were the source of infection for human outbreaks and positive horses detected at the slaughterhouse). In this period of time, approximately 7 million horses have been consumed in the European Union; thus the 28 infected animals detected in Europe, represent a prevalence of only 4.1 million slaughtered horses. The fact that all of the infected horses were imported from countries with a high prevalence of *Trichinella* sp. infection in pigs and or wildlife suggests that there is a close relationship between the infection in these animals and the horse infection (Pozio, 2001a; Murrell *et al.*, 2004).

Epidemiological investigations of the five most recent human outbreaks have shown that they occurred because of inadequate veterinary controls at the slaughterhouse. Horse-meat outbreaks have important consequences for public health because of the high number of infected persons resulting from consumption of meat from a single horse and the very severe symptomatology, at times resulting in death. This has a high impact in terms of medical costs, horse-meat market economics, which collapses after each outbreak, and in legal and administrative terms related to the implementation of control measures at the national and international level (Ancelle, 1998).

In spite of several epidemiological surveys performed at the point of origin of the infected horses (Murrell *et al.*, 2004; Pozio, E., unpublished data), more evidence on the epidemiology of equine trichinellosis is needed. Recent investigations have shown a relationship between *Trichinella* sp. infection in horses and the local prevalence of pig infections, and the feeding of animal meat scraps to improve horse condition prior to sale; although considered herbivores, 32% of horses tested ate meat when offered (Murrell *et al.*, 2004). In the four cases where sylvatic species of *Trichinella* (*T. britovi* or *T. murrelli*) have been detected, an association between infection in horses and wildlife or fur-reared animals has also been postulated (Pozio, 2001a).

The feeding of animal products to horses is a practice that occurs in several countries, including those of origin of infected horses (e.g. Serbia) (Murrell *et al.*, 2004). The increasing reports of human outbreaks of trichinellosis in France and Italy in the 1990s, and the detection of *Trichinella*-infected horses at slaughter occurred during a period in eastern European countries when there was a widespread breakdown in veterinary control services (Murrell and Pozio, 2000; Olteanu, 2001;

Djordjevic *et al.*, 2003; Cuperlovic *et al.*, 2005). The presence of thin capsules around the larvae in muscle tissues of the horses slaughtered in January and the presence of thick capsules in the larvae from horses slaughtered in April and October seem to support the hypothesis that horses acquire this infection in late autumn or winter, i.e. when most of the backyard pigs are slaughtered at home, which in Europe is legal without any veterinary control if pork is for own consumption. Almost uniformly, infections in humans occur where there is either no veterinary control over meat hygiene or the service in place is inefficient (Olteanu, 2001; Djordjevic *et al.*, 2003, 2005).

6.3. Trichinellosis in Humans

Trichinella sp. infections in humans are related to cultural food practices, which include dishes based on raw or undercooked meat of different animal origins (Table 3). The presence of the parasite in domestic and/or wild animals is not a sufficient risk in itself, however, for the infection to occur in the human population. For example, in Finland, where there is a high prevalence of infection in animals, no infection has been documented in humans, probably due to the practice of eating only well-cooked meat (Pozio, 1998). Similarly, in most African countries south of the Sahara, human infection is seldom documented in spite of the presence of sylvatic *Trichinella* sp., because about a third of all African populations are of the Bantu ethnic group, which rarely consumes meat.

Overall, the most important source of *Trichinella* sp. infection for humans remains pork and its related products from domestic pigs. Important foci of human trichinellosis from pork occur in Central (Mexico) and South America (Argentina and Chile) (Ortega Pierres *et al.*, 2000; Ribicich *et al.*, 2005), Asia (China, Laos, Myanmar, Thailand, Vietnam) (Takahashi *et al.*, 2000; Pozio, 2001b; Liu and Boireau, 2002) and Europe (Bosnia-Herzegovina, Bulgaria, Byelorussia, Croatia, Georgia, Latvia, Lithuania, Poland, Romania, Russia, Serbia, Spain and Ukraine) (Pozio, 2001a; Pozio and Zarlenga, 2005).

In conclusion, raw or undercooked meat from carnivore and omnivore mammals, birds and reptiles pose an important risk for

Table 3 Animals, besides domestic pigs, which were the source of trichinellosis

Meat origin	Country	References
Wild boar (<i>Sus scrofa</i>)	Europe, North and South America, Asia	Nadzhimiddinov <i>et al.</i> , 1965; Boev <i>et al.</i> , 1970; Khamboonruang, 1991; Eisenman and Einat, 1992; Jongwutiwes <i>et al.</i> , 1998; Pozio, 1998; Roy <i>et al.</i> , 2003; Ribicich <i>et al.</i> , 2005
Wild pig (hybrid between <i>Sus scrofa vittatus</i> and <i>Sus celebensis</i>)	Papua New Guinea	Owen <i>et al.</i> , 2001, 2005
Warthog (<i>Phacochoerus africanus</i>)	Ethiopia, Senegal, Tanzania	Gretillat and Vassiliades, 1967; Bura and Willett, 1977; Perdomo Gonzales <i>et al.</i> , 1986; Kefenie <i>et al.</i> , 1988; Kefenie and Bero, 1992; Pozio <i>et al.</i> , 1994b
Bush pig (<i>Potamochoerus larvatus</i>)	Kenya	Nelson, 1970; Pozio <i>et al.</i> , 1994b
Walrus (<i>Odobenus rosmarus</i>)	Canada and Greenland	Proulx <i>et al.</i> , 2002; Serhir <i>et al.</i> , 2001
Black bears (<i>Ursus americanus</i>)	USA, Canada	Nelson <i>et al.</i> , 2003; Roy <i>et al.</i> , 2003; Schellenberg <i>et al.</i> , 2003
Brown bears (<i>Ursus arctos</i>)	Alaska, Canada, China, Bulgaria, Russia, Siberia	CDC, 1981; Shiota <i>et al.</i> , 1999; Peklo <i>et al.</i> , 2002
Cougar (<i>Felis concolor</i>)	USA, Argentina	Dworkin <i>et al.</i> , 1996; Ribicich <i>et al.</i> , 2005
Badger (<i>Meles meles</i>)	Korea, Bulgaria, Russia	Suzdaltsev <i>et al.</i> , 1999; Sohn <i>et al.</i> , 2000; ProMed mail 2005
Red fox (<i>Vulpes vulpes</i>)	Italy	Pozio <i>et al.</i> , 2001e
Jackal (<i>Canis aureus</i>)	Thailand, Algeria	Khamboonruang, 1991; Nezri <i>et al.</i> , 2005
Armadillo (<i>Chaetophractus villosus</i>)	Argentina	Ribicich <i>et al.</i> , 2005
Squirrel	Thailand	Khamboonruang, 1991
Monitor lizard (<i>Varanus nebulosus</i>)	Thailand	Khamboonruang, 1991
Turtle	Thailand	Khamboonruang, 1991
Horse	France, Italy	Ancelle, 1998; Pozio and Zarlenga, 2005
Beef, mutton, goat*	China	Murrell, 1994; Takahashi <i>et al.</i> , 2000
Dogs	China, Russia, Slovak Republic	Takahashi <i>et al.</i> , 2000; Dubinsky <i>et al.</i> , 2001; ProMed mail 2004

*Meat from these animals has been implicated as a source of infection for humans, even if experimental infections have shown that cattle, sheep and goats can acquire a transient infection (Campbell, 1983b; Smith *et al.*, 1990; Reina *et al.*, 1996; Theodoropoulos *et al.*, 2000). It could be argued that sheep, goats and cattle could acquire *T. spiralis* infection when they are bred in areas that are highly endemic for *T. spiralis* infection in pigs, but this must be supported by further epidemiological investigations.

Trichinella sp. transmission to humans. Except for *T. zimbabwensis*, *Trichinella* T8 and T9, all species and genotypes of *Trichinella* have been detected in humans; based on animal experiment data, it is likely these exceptions are also infective for humans.

6.3.1. *Changing Patterns of the Epidemiology of Human Infections*

In the United States, Canada and European Union countries, human infections due to the consumption of pork from domestic pigs have nearly or completely disappeared because of both the improvement of pig-production facilities and practices and the improvement of detection technologies employed in the slaughterhouses. In these countries, the occasional infections with *T. spiralis* that occur are related to the consumption of pork from the so-called backyard pigs or pigs reared on organic farms (Pozio, 1998). However, a large biomass of the parasite continues to exist in developing countries of Central and South America, Europe and Asia, where an increase in human population movement to urban areas is occurring in China, or even to industrialized countries is seen. This important demographic factor has resulted in new and different patterns of human infections. For example, trichinellosis is emerging in some urban areas in China where affluence has increased the demand for pork, particularly, in dishes that traditionally may not be well-cooked (meat dumplings) (Wang *et al.*, 1998).

The migration of persons from eastern Europe to the European Union (EU), mainly for work, has led to an increase in the quantity of pork products sent from these countries to the EU as Christmas gifts or brought back to the EU by expatriates visiting their country of origin for the holidays. This behavior has resulted in several human outbreaks of trichinellosis in Germany, Italy and the United Kingdom (Pozio and Marucci, 2003). Since human trichinellosis is quite rare in many EU countries, many local physicians are not familiar with the disease and experience problems in diagnosing it. Delays in diagnosis and treatment favor the establishment of larvae in muscles and the development of a collagen capsule, which render the larvae resistant to drugs (Pozio *et al.*, 2001g, 2003; Dupouy-Camet *et al.*, 2002). In

addition to constituting a risk to human health, imported pork products infected with *T. spiralis* threaten the pig industry in the EU, particularly for organic farms which typically practice free-range pasturing of pigs, and which may have a risk due to the spread in the environment of infected pork scraps. For example, *T. spiralis* is not present in domestic or wild animals of Italy or Switzerland (Pozio, 2001a), and an accidental exposure to infected products could introduce a new pathogen into the food supply of these countries.

In some cases, human migration has resulted in the introduction of new food practices and dishes based on raw or undercooked pork or pork products, which have led to trichinellosis outbreaks among the unaware immigrant communities in endemic countries, especially for migrants from Cambodia, Laos, Thailand and Vietnam; those in the United States and Israel, where the control for *Trichinella* infection in domestic pigs and wild boars is not compulsory, are especially at risk (Imperato *et al.*, 1974; Stehr-Green and Schantz, 1986; McAuley *et al.*, 1992; Graves *et al.*, 1996; Marva *et al.*, 2005).

In China, the "Western Region Development of China" strategy implemented in 1990s, elicited migration and settlement of large numbers of people from the central to the western areas, which led in turn to an increased quantity of pork products being taken from central to western regions, either commercially or privately. The areas of central China where potentially infected meat can be exported to other provinces have prevalence rates of *T. spiralis* infection in pigs of 6.8% in Hubei and 4.3% in Henan (Wang and Cui, 2001). In the new western communities which are likely to import pork, pigs are being reared under relatively primitive conditions in which the animals are exposed frequently to raw waste meat scraps, and are free to scavenge animal carcasses from both wild and domestic sources (Cui *et al.*, 2006). This has led to a dramatic increase in the size of the human population at risk for trichinellosis in the western areas of China (Cui *et al.*, 2006). In 1990, the incidence of *Trichinella* infection in examined pork samples from the markets in Xing City (Qinghai province, western China) was 0.1%, but was 15.9% in Huangyuan county in 1997 (Tibet Autonomous Prefecture, Qinghai province, western China), and 23.0% in Delingha city in 2004 (Qinghai Province, western China). These infection rates also pose a potential danger to the rapidly developing tourist

industry. In fact, the increasing demand for meat for tourist hotels and restaurants has led to a rapid increase in the number of small farms where pigs are often fed with swills containing raw pork scraps from the restaurants or hotels, thereby providing a mechanism for the amplification of the transmission cycle (Cui *et al.*, 2006).

The increasing number of international travelers has resulted in many reports of tourists who acquired *Trichinella* sp. infections while traveling or hunting in endemic areas and later developed the clinical disease after their return to their home countries. In most instances, diagnosis was difficult because the infections appeared as isolated cases (McAuley *et al.*, 1991; Dupouy-Camet *et al.*, 1998; Shiota *et al.*, 1999; Nakamura *et al.*, 2003). Trichinellosis in travelers has occurred after the consumption of pork from a warthog in Africa (Dupouy-Camet *et al.*, 1998); bear meat in Canada and Greenland (Nozais *et al.*, 1996; Dupouy-Camet *et al.*, 1998; Ancelle *et al.*, 2005), pork in China, Egypt, Indonesia (Bali Island), Laos and Malaysia (Therizol *et al.*, 1975; De Carneri and Di Matteo, 1989; Shiota *et al.*, 1999; Kurup *et al.*, 2000; ICT); and wild boar meat in Turkey and Algeria (Niquet *et al.*, 1979; Michel *et al.*, 1986).

In countries where most of the population follows the Muslim or Judaism religious law, the consumption of pork and the meat of carnivores is forbidden, therefore, *Trichinella* sp. infection is seldom documented in humans. However, the increasing secularism, demographic changes and the presence of populations with different religions in these countries, along with the increasing tourism they are experiencing, has stimulated an increase in pig production, and the consumption of game meat, which is not subject to veterinary control because officially "pigs" do not exist. In Turkey, three outbreaks of trichinellosis occurred from the consumption of pork in Antalya (more than 40 people), Bursa (seven people) and Izmir (more than 600 people) between 2003 and 2004 (Heper *et al.*, 2005; Ozdemir *et al.*, 2005; Pozio and Zarlenga, 2005; Turk *et al.*, 2006). In Israel, six small outbreaks occurred mostly in the Christian Arab population from the consumption of pork from wild boars prior to 1997 (Eisenman and Einat, 1992; Marva *et al.*, 2005). In Lebanon and Syria, large outbreaks have also occurred in Christian villages from the consumption of pork from wild boars (Olaison and Ljungstrom,

1992; Hallaj, Z., personal communication). In Algeria, six small outbreaks (involving a total of 51 people) occurred from the consumption of a domestic pig (Gerard, 1946) and wild boars (Lanoire *et al.*, 1963; Verdaguer *et al.*, 1963; Mémin *et al.*, 1968; Barabe *et al.*, 1977; Michel *et al.*, 1986), and a single case due to the consumption of meat from a jackal (Nezri *et al.*, 2006). In Ethiopia, where the Coptic religion is professed by 35–40% of the inhabitants, trichinellosis has been frequently occurring from the consumption of warthog (Perdomo Gonzales *et al.*, 1986; Kefenie *et al.*, 1988; Kefenie and Bero, 1992; Gelnew, T., personal communication).

7. A NEW APPROACH: *TRICHINELLA*-FREE AREAS OR FARMS. IS IT POSSIBLE?

In recent years, debate has intensified over the validity of the concept of *Trichinella*-free areas (Pozio, 1998; European Food Safety Authority, 2005). This has great economic importance because of the high cost associated with testing of all slaughtered pigs from the area for *Trichinella*. In countries, where pig production is carried out using good rearing standards (Gamble *et al.*, 2000) and humans do not consume raw pork products, *Trichinella* infection in either humans or pigs has not been documented for a long time (e.g. Denmark, The Netherlands). Unfortunately, the absence of reports of *Trichinella* infections in these regions tends to lead to complacency among consumers and producers, with a lessening of appreciation for the risk of this zoonosis and a relaxation of veterinary control measures at slaughter. As an example, until recently in Ireland no infections had been documented in either humans or animals for 34 years, suggesting that the country was *Trichinella*-free (Rafter *et al.*, 2005). This in turn resulted in a reduction in slaughterhouse control measures where the pig testing rate dropped to 20%. However, an epidemiological survey carried out on red foxes revealed the presence of *T. spiralis*-infected animals in countries thought to be *Trichinella*-free for this period, clearly demonstrating that the sylvatic cycle can flourish independent of the domestic cycle (Rafter *et al.*, 2005). The long-term survival of *Trichinella* in foxes of Ireland may be explained in part by hunters

leaving the carcasses in the field after skinning. In Ireland, the high humidity and low temperatures during the hunting season, i.e. autumn and winter, favor the survival of larvae and allow for the transmission of *Trichinella* through a fox-fox cycle. It can be considered, that the lack of positive reports among domestic pigs may be related to the fact that random routine testing is conducted on relatively few pigs, rather than to the existence of a barrier between the sylvatic and the domestic cycle. The lack of human infections may simply be due to the fact that people of this region tend to cook pork well.

In New Zealand, where *T. spiralis* and its hosts such as pigs and rats were imported during the European colonization, the comprehensive control for *Trichinella* infection in pigs is only compulsory for exported meat and only a random sampling from 300 mature pigs is performed at slaughter for products destined for the domestic market. This sampling size is based statistically on the expectation that the inspection method would detect *Trichinella* at a prevalence of 0.5%. Clearly, this is a minimalist approach, and it may not prevent transmission of the infection to animals or humans. In fact, *Trichinella* infections have been repeatedly documented in domestic pigs in New Zealand in 1965, 1968, 1974, 1997 and 2001; brown rats in 1965 and 2001; cats in 1965, 1974 and 2001 (Buncic, 1997; Paterson *et al.*, 1997; E. Pozio, unpublished data); and humans up to 2001 (Liberona and MacDiarmid, 1988; ICT).

In Tasmania, *T. pseudospiralis* has been detected in marsupials and birds, beginning in 1990 (Obendorf *et al.*, 1990). Australia, however, has always been considered *Trichinella*-free, but this status is based not on any extensive epidemiological investigation on wildlife, but on limited investigations on synanthropic rats, and domestic cats and pigs (Waddell, 1969) and on examination of tongues from 45 dingoes and 22 red foxes from Victoria and New South Wales in 2001, all of which were negative by artificial digestion (Jenkins D. and Pozio, E., unpublished data). There is not a documented autochthonous case in humans. But because *T. papuae* is widespread in wild pigs and saltwater crocodiles of Papua New Guinea, which is very close to Australia (less than 180 km of sea separate the two countries) and the saltwater crocodile is also a marine animal, the status as *Trichinella*-free should be modified until more extensive searches have been conducted.

Mediterranean islands are also considered to be *Trichinella*-free for the lack of reports in animals or humans, but because these locations lack veterinary control at slaughter, the status of pigs is uncertain. To verify whether or not the island of Corsica can be considered as *Trichinella*-free, a survey of domestic pigs was conducted at slaughterhouses in 2004. Surprisingly, *T. britovi* was detected in pigs and in the surrounding wildlife (wild pigs and a fox) (Boireau and Valleé, 2004). In Sardinia, no control has been done at the slaughterhouse due to a conviction that the island was *Trichinella*-free, which was based on the examination of a very low number of domestic and sylvatic animals. However, in 2005, an outbreak of trichinellosis involving 11 people resulted from the consumption of raw pork infected with *T. britovi* from a free-range reared sow (Pozio *et al.*, submitted). This proves the validity of the belief that the declaration as a *Trichinella*-free area must be based on extensive animal surveys and surveillance, preferably at slaughter.

These examples demonstrate how the lack of reports of infection among domestic animals and humans over an extended timeframe cannot be considered as a justification for area-wide *Trichinella*-free status. All self-declared *Trichinella*-free areas should institute a sustaining monitoring program, which includes not only domestic animals but also wildlife.

However, an alternative approach that awards certification to farms with sustained *Trichinella*-free status is being seriously studied in some countries, most notably in the United States and in the European Union (Pozio, 1998; Pyburn *et al.*, 2005; Kapel, 2005). An extensive pilot program, The U.S. Trichinae Certification Program, under the supervision of the U.S. Department of Agriculture, involving over 450 pig farms, is in progress (Pyburn *et al.*, 2005). These farms undergo a pre-certification site audit for risk factors and infection status of the pig herd. Subsequently, the farm is subjected to both scheduled and unscheduled audits and testing of pigs to ensure the herd remains *Trichinella*-free. Pork products from these certified herds can be marketed as such, with the anticipated commercial benefits associated with selling safe pork. The progress and results of this trial will be watched with interest.

8. CONCLUDING REMARKS

It was the authors' intent in undertaking this review of *Trichinella* to highlight aspects of the parasite's biology and epidemiological features that are of importance to those with basic research interests and to public health efforts. Although trichinellosis has declined significantly as a zoonosis, due chiefly to a reduction of domestic trichinellosis in developed countries, it remains a potential risk because of the continuing presence of most species of *Trichinella* as a parasite of wild animals. Further, the strong opportunism associated with this parasite, with its broad reservoir host range and diverse transmission features makes the potential for its re-emergence whenever the food-safety barriers are weakened by socioeconomic events. Examples are seen in the equine trichinellosis outbreaks in Europe, and the reemergence of porcine trichinellosis in countries undergoing major political and economic change (e.g. former Yugoslavia, Romania and Argentina). It behooves veterinary and public health agencies to become well acquainted with the causes of the re-emergence of trichinellosis in countries where its formerly successful control measures for this zoonosis was placed in jeopardy or severely compromised by these political, economic and agricultural changes. It is important then, that the systematics and ecology of all species including both, the classical domestic *T. spiralis* and the sylvatic species, which are increasingly implicated as a cause of human trichinellosis or a significant risk, be well understood.

The emphasis placed in this review, therefore, is the knowledge gained in recent years on the basic biology, especially evolutionary biology, ecology, and biogeography of *Trichinella* spp. It should be apparent that our current knowledge of these aspects represents only the barest of essentials pertaining to these parasites. In a sense, we are only at the beginning of the journey to full knowledge, and that much more research is needed to be carried out before the gaps are filled. Those interested in pursuing these questions can look forward to a demanding task, but they can also take comfort in having a good platform to build upon the availability of molecular tools for genetic analysis and a large, available collection of isolates and biogeographic knowledge residing in the International *Trichinella* Reference Centre. If another review on the systematics and epidemiology of *Trichinella* is undertaken in a

decade or so, it will likely describe much new knowledge and perhaps provide a surprising new view of this species complex. We certainly hope so!

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REFERENCES

- Ancelle, T. (1998). History of trichinellosis outbreaks linked to horse meat consumption 1975–1998. *Euro Surveillance* **3**, 86–89.
- Ancelle, T., Dupouy-Camet, J., Bournoux, M.E., Fourestie, V., Petit, H., Mougeot, G., Nozais, J.P. and Lapierre, J. (1988). Two outbreaks of trichinosis caused by horsemeat in France in 1985. *American Journal of Epidemiology* **127**, 1302–1311.
- Ancelle, T., De Bruyne, A., Poisson, D. and Dupouy-Camet, J. (2005). Outbreak of trichinellosis due to consumption of bear meat from Canada, France, September 2005. *Eurosurveillance* **10**, 10.
- Andrews, J.R.H., Bandi, C., Pozio, E., Gomez Morales, M.G., Ainsworth, R. and Abernethy, D. (1995). Identification of *Trichinella pseudospiralis* from a human case using random amplified polymorphic DNA. *American Journal of Tropical Medicine and Hygiene* **53**, 185–188.
- Appleton, J.A. and Romaris, F. (2001). A pivotal role for glycans at the interface between *Trichinella spiralis* and its host. *Veterinary Parasitology* **101**, 249–260.
- Appleyard, G.D., Zarlenga, D., Pozio, E. and Gajadhar, A.A. (1999). Differentiation of *Trichinella* genotypes by polymerase chain reaction using sequence-specific primers. *Journal of Parasitology* **85**, 556–559.
- Arakawa, A. and Todd, A.C. (1971). Comparative development of temperate zone and arctic isolates of *Trichinella spiralis* in the white mouse. *Journal of Parasitology* **57**, 526–530.

- Bandi, C., La Rosa, G., Bardin, M.G., Damiani, G., Comincini, S., Tasciotti, L. and Pozio, E. (1995). Random amplified polymorphic DNA fingerprints of the eight taxa of *Trichinella* and their comparison with allozyme analysis. *Parasitology* **110**, 401–407.
- Bandi, C., La Rosa, G., Comincini, S., Damiani, G. and Pozio, E. (1993). Random amplified polymorphic DNA technique for the identification of *Trichinella* species. *Parasitology* **107**, 419–424.
- Barabe, P., Motte, M., Larbi, M., Thomas, D., Bobin, P. and Foullon, X. (1977). La trichinose aiguë, à propos de deux observations algériennes. *Médecine et armées* **5**, 37–40.
- Barakat, R.M., El-Sawy, M.F., Selim, M.K. and Rashwan, A. (1982). Trichinosis in some carnivores and rodents from Alexandria, Egypt. *Journal of the Egyptian Society of Parasitology* **12**, 445–451.
- Batkaev, A.I. and Vakker, V.G. (1992). The role of corsac in circulation of trichinelles in the Middle Irtysh territory. *Proceedings of the 6th Scientific Conference on Trichinellosis*, pp. 24–26. Kirov, Moscow.
- Bessonov, A.S. (1981). Changes in the epizootic and epidemic situation of trichinellosis in the U.S.S.R. In: *Trichinellosis* (C.W. Kim, E.J. Ruitenberg and J.S. Teppema, eds), pp. 365–368. Chertsey, England: Reedbooks.
- Bessonov, A.S., Penkova, R.A. and Gumenshchikova, V.P. (1978). *Trichinella pseudospiralis* Garkavi, 1972: Morphological and biological characteristics and host specificity. In: *Trichinellosis* (C.W. Kim and Z.S. Pawlowski, eds), pp. 79–93. Hanover, New Hampshire: University Press of New England.
- Bianli, X., Zaolin, C., Quingxia, H. and Hui, L. (2001). Epidemiological survey of *Trichinella* infection in some areas of Henan Province. *Parasite* **8**(S2), S71–S73.
- Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T. and Thomas, W.K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**, 71–75.
- Boev, S.N., Bondareva, V.I., Sokolova, I.B. and Tazieva, Z.H. (1970). A study on the distribution of trichinellosis in Kazakhstan. *Wiadomosci Parazytologiczne* **16**, 74–75.
- Boev, S.N., Britov, V.A. and Orlov, I.V. (1979). Species composition of Trichinellae. *Wiadomosci Parazytologiczne* **25**, 495–503.
- Boireau, P. and Vallée, I. (2004). Epidemiologie des trichinelloses animales en France. Surveillance officielle et surveillance sérologique. *Report for the French Ministry of Agriculture*, pp. 1–39, 5 April 2004.
- Boireau, P., Vallee, I., Roman, T., Perret, C., Mingyuan, L., Gamble, H.R. and Gajadhar, A. (2000). *Trichinella* in horses: a low frequency infection with high human risk. *Veterinary Parasitology* **93**, 309–320.
- Bouree, P., Bouvier, J.B., Passeron, J., Galanaud, P. and Dormont, J. (1979). Outbreak of trichinosis near Paris. *British Medical Journal* **1**, 1047–1049.

- Bowen, G.J., Clyde, W.C., Koch, P.L., Ting, S., Alroy, J., Tsubamoto, T., Wang, Y. and Wang, Y. (2002). Mammalian dispersal at the Paleocene-Eocene boundary. *Science* **295**, 2062–2065.
- Britov, V.A. (1969). Some differences between natural and synanthropic strains of *Trichinella*. *Wiadomosci Parazytologiczne* **15**, 555–560.
- Britov, V.A. (1971). Biologic methods of determining *T. spiralis* Owen, 1835 varieties. *Wiadomosci Parazytologiczne* **17**, 477–480.
- Britov, V.A. (1982). *Trichinellosis agents*. Moscow, Nauka [in Russian].
- Britov, V.A. (1994). *Trichinella* as an object of experimental biology. In: *Trichinellosis* (C.W. Campbell, E. Pozio and F. Bruschi, eds), pp. 141–146. Rome, Italy: Istituto Superiore di Sanità Press.
- Britov, V.A. (1997). Trichinellosis in Kamchatka. *Wiadomosci Parazytologiczne* **43**, 287–288.
- Britov, V.A. and Boev, S.N. (1972). Taxonomic rank of various strains of *Trichinella* and their circulation in nature. *Vestnik Akademii Nauk KSSR* **28**, 27–32.
- Bruschi, F. (2002). The immune response to the parasitic nematode *Trichinella* and the ways to escape it. From experimental studies to implications for human infection. *Current Drug Targets Immune Endocrine Metabolic Disorders* **2**, 269–280.
- Bruschi, F., Pozio, E., Watanabe, N., Gomez Morales, M.A., Ito, M., Huang, Y. and Binaghi, R. (1999). Anaphylactic response to parasite antigens: IgE and IgG1 independently induce death in *Trichinella*-infected mice. *International Archives of Allergy and Immunology* **119**, 291–296.
- Buncic, S. (1997). A case of a pig infected with *Trichinella spiralis*. *Surveillance* **24**, 8.
- Bura, M.W. and Willett, W.C. (1977). An outbreak of trichinosis in Tanzania. *East African Medical Journal* **54**, 185–193.
- Burton, J. and Pearson, B. (1987). *Rare Mammals of the World*. Lexington, MA: Stephen Greene Press.
- Calero, R., Martinez, F., Hernandez, S. and Acosta, I. (1978). Parasitacion de *Buteo buteo* (Aves: Accipitridae) por *Trichinella* sp. en el parque zoológico de Jerez de la Frontiera. *Revista Iberica de Parasitologia* **38**, 135–138.
- Campbell, W.C. (1983a). Historical introduction. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 1–30. New York and London: Plenum Press.
- Campbell, W.C. (1983b). Modes of transmission. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 425–444. New York and London: Plenum Press.
- Campbell, W.C. (1991). *Trichinella* in Africa and the *nelsoni* affair. In: *Parasitic Helminthes and Zoonoses in Africa* (C.N.L. Macpherson and P.S. Craig, eds), pp. 83–100. London: Unwin Hyman.
- Castro, G.A. (1997). Mucosal adaptation to invasion by *Trichinella spiralis*: a lesson in integrative biology. In: *Trichinellosis ICT9* (G. Ortega-Pierres,

- R. Gamble, F. van Knapen and D. Wakelin, eds), pp. 169–173. D.F. México: Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional México.
- Centers for Disease, Control. (1981). Trichinosis associated with meat from a grizzly bear-Alaska. *Morbidity Mortality Weekly Report* **30**, 115–116 121.
- Chambers, A.E., Almond, N.M., Knight, M., Simpson, A.J. and Parkhouse, R.M. (1986). Repetitive DNA as a tool for the identification and comparison of nematode variants: application to *Trichinella* isolates. *Molecular and Biochemical Parasitology* **21**, 113–120.
- Cironeanu, I. (1974). Trichinellosis in domestic and wild animals in Romania. In: *Trichinellosis* (C.W. Kim, ed.), pp. 549–555. New York: Intext Educational Publishers.
- Correa, D., Naro Robles, J., De La Rosa, J.L. and Ortega Pierres, M.G. (1997). Trichinellosis: an overview of research in México: 1891–1996. In: *Trichinellosis ICT9* (G. Ortega-Pierres, R. Gamble, F. van Knapen and D. Wakelin, eds), pp. 1–7. D.F. México: Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional México.
- Csokor, J. (1884). Experimentelle infection eines Pferdes mit Trichinen. *Allgemein Wien Medizin Zeitung* **29**, 248.
- Cui, J., Wang, Z.Q. and Kennedy, M.W. (2006). Risk of re-emergence of trichinellosis in China. *Trends in Parasitology* **22**, 54–55.
- Cuperlovic, K., Djordjevic, M. and Pavlovic, S. (2005). Re-emergence of trichinellosis in southeastern Europe due to political and economic changes. *Veterinary Parasitology* **132**, 159–166.
- Dame, J.B., Murrell, K.D., Worley, D.E. and Schad, G.A. (1987). *Trichinella spiralis*: genetic evidence for synanthropic subspecies in sylvatic hosts. *Experimental Parasitology* **64**, 195–203.
- Daszak, P., Cunningham, A.A. and Hyatt, A.D. (2000). Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science* **287**, 443–449.
- De Carneri, I. and Di Matteo, L. (1989). Epidemiology of trichinellosis in Italy and in neighboring countries. *Annali Istituto Superiore di Sanità* **25**, 625–633 [in Italian].
- Despommier, D.D. (1983). Biology. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 75–151. New York and London: Plenum Press.
- Despommier, D.D. (1990). *Trichinella spiralis*: the worm that would be virus. *Parasitology Today* **6**, 193–196.
- Dick, T.A. (1983). Species, and infraspecific variation. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 31–73. New York and London: Plenum Press.
- Dick, T.A. and Belosevic, M. (1978). Observations on a *Trichinella spiralis* isolate from a polar bear. *Journal of Parasitology* **65**, 1143–1145.

- Dick, T.A. and Pozio, E. (2001). *Trichinella* spp. and trichinellosis. In: W.M. Samuel, M.J. Pybus, A.A. Kocan, (eds), *Parasitic Diseases of Wild Mammals*, 2nd Edition., (pp. 380–396). Ames: Iowa State University Press.
- Dick, T.A., Lu, M.C., deVos, T. and Ma, K. (1992). The use of the polymerase chain reaction to identify porcine isolates of *Trichinella*. *Journal of Parasitology* **78**, 145–148.
- Djordjevic, M., Bacic, M., Petricevic, M., Cuperlovic, K., Malakauskas, A., Kapel, C.M. and Murrell, K.D. (2003). Social, political, and economic factors responsible for the reemergence of trichinellosis in Serbia: a case study. *Journal of Parasitology* **89**, 226–231.
- Djordjevic, M., Cuperlovic, K., Savic, M. and Pavlovic, S. (2005). The need for implementation of International Commission on Trichinellosis recommendations, quality assurance standards, and proficiency sample programs in meat inspection for trichinellosis in Serbia. *Veterinary Parasitology* **132**, 185–188.
- Dubinsky, P., Stefancikova, A., Kincekova, J., Ondriska, F., Reiterova, K. and Medvedova, M. (2001). Trichinellosis in the Slovak Republic. *Parasite* **8**(S2), S100–S102.
- Dupouy-Camet, J. (2000). Trichinellosis: a worldwide zoonosis. *Veterinary Parasitology* **93**, 191–200.
- Dupouy-Camet, J., Allegretti, S. and Truong, T.P. (1998). Enquete sur l'incidence de la trichinellose en France (1994–1995). *Bulletin Epidemiologique Hebdomadaire* **28**, 122–123.
- Dupouy-Camet, J., Kociecka, W., Bruschi, F., Bolas-Fernandez, F. and Pozio, E. (2002). Opinion on the diagnosis and treatment of human trichinellosis. *Expert Opinion in Pharmacotherapy* **3**, 1117–1130.
- Dworkin, M.S., Gamble, H.R., Zarlenga, D.S. and Tennican, P.O. (1996). Outbreak of trichinellosis associated with eating cougar jerky. *Journal of Infectious Diseases* **174**, 663–666.
- Eisenman, A. and Einat, R. (1992). A family outbreak of trichinosis acquired in Israel. *Harefuah* **122**, 702–704, 751 [in Hebrew].
- European Food Safety Authority. (2005). Opinion of the scientific panel on Biological Hazards on the “Request for an opinion on the feasibility of establishing *Trichinella*-free areas, and if feasible on the risk increase to public health of not examining pigs from those areas for *Trichinella* spp. pp. 1–40.
- Fay, F.H. (1968). Experimental transmission of *Trichinella spiralis* via marine amphipods. *Canadian Journal of Zoology* **46**, 597–599.
- Flockhart, H.A., Harrison, S.E., Dobinson, A.R. and James, E.R. (1982). Enzyme polymorphism in *Trichinella*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**, 541–545.
- Forbes, L.B. (2005). Zoonotic aspects of trichinellosis. In: *Marine Parasitology* (K. Rhode, ed.), pp. 436–439. Melbourne, Australia: CSIRO.

- Frazier, I. (2005). *Hogs wild*. *The New Yorker*, December, 12, pp. 71–83.
- Fukumoto, S., Nagai, D., Yazaki, S., Kamo, H. and Yamaguchi, T. (1988). The molecular phylogenetic tree of the genus *Trichinella* constructed from isozyme patterns. *Parasitology Research* **74**, 574–580.
- Fukumoto, S., Takechi, M., Kamo, H. and Yamaguchi, T. (1987). Comparative studies on soluble protein profiles and isozyme patterns of seven *Trichinella* isolates. *Parasitology Research* **73**, 352–357.
- Gajadhar, A., Forbes, L. and Steeves-Gurnsey, T. (2004). The occurrence and distribution of *Trichinella* spp. in Canadian wildlife. *11th International Conference on Trichinellosis*, abstract No. 66. San Diego, CA, 8–12 August.
- Gamble, H.R., Bessonov, A.S., Cuperlovic, K., Gajadhar, A.A., van Knapen, F., Noeckler, K., Schenone, H. and Zhu, X. (2000). International commission on trichinellosis: recommendations on methods for the control of *Trichinella* in domestic and wild animals intended for human consumption. *Veterinary Parasitology* **93**, 393–408.
- Gamble, H.R., Pozio, E., Lichtenfels, J.R. and Zarlenga, D.S. (2005). *Trichinella pseudospiralis* from a wild pig in Texas. *Veterinary Parasitology* **132**, 147–150.
- Garkavi, B.L. (1972). Species of *Trichinella* isolated from wild animals. *Veterinariya* **10**, 90–91.
- Gasser, R.B., Hu, M., Abs El-Osta, Y.G., Zarlenga, D.S. and Pozio, E. (2004). Non-isotopic single-strand conformation polymorphism analysis of sequence variability in ribosomal DNA expansion segments within the genus *Trichinella* (Nematoda: Adenophorea). *Electrophoresis* **25**, 3357–3364.
- Gasser, R.B., Zhu, X.Q., Monti, J.R., Dou, L., Cai, X. and Pozio, E. (1998). PCR-SSCP of rDNA for the identification of *Trichinella* isolates from mainland China. *Molecular and Cellular Probes* **12**, 27–34.
- Gaugusch, Z. (1950). Studies on the infection of poikilothermic animals with *T. spiralis*. *Veterinary Medicine* **5**, 95–106.
- Gerard, R. (1946). Petite épidémie de trichinose en Algérie. *Revue Médecine Navale* **1**, 355–362.
- Gerlach, A.C. (1873). *Die Trichinen*. Hannover, Germany: Schmorl and von Seefeld.
- Gomez Morales, M.A., Mele, R., Sanchez, M., Sacchini, D., De Giacomo, M. and Pozio, E. (2002). Increased CD8⁺-T-cell expression and a type 2 cytokine pattern during the muscular phase of *Trichinella* infection in humans. *Infection and Immunity* **70**, 233–239.
- Gomez Villafane, I.E., Minarro, F., Ribicich, M., Rossetti, C.A., Rossotti, D. and Busch, M. (2004). Assessment of the risks of rats (*Rattus norvegicus*) and opossums (*Didelphis albiventris*) in different poultry-rearing areas in Argentina. *Brazilian Journal of Microbiology* **35**, 359–363.
- Gottstein, B., Pozio, E., Connolly, B., Gamble, H.R., Eckert, J. and Jakob, H.P. (1997). Epidemiological investigation of trichinellosis in Switzerland. *Veterinary Parasitology* **72**, 201–207.

- Gould, S.E. (1970). History. In: *Trichinosis in man and animals* (S.E. Gould, ed.), pp. 3–18. Springfield, IL: Charles C. Thomas Publisher.
- Graves, T., Harkess, J. and Crutcher, J.M. (1996). Case report: locally acquired trichinosis in an immigrant from Southeast Asia. *Journal of Oklahoma State Medical Association* **89**, 402–404.
- Gretillat, S. and Vassiliades, G. (1967). Presence of *Trichinella spiralis* (Owen, 1835) in wild carnivora and swine of the region of the Senegal river basin. *Comptes rendus hebdomadaires des seances de l'Academie des Sciences* **264**, 1297–1300.
- Guevara Pozo, D. and Contreras-Pena, A.J. (1966). *Trichinella spiralis* (Owen, 1835): experiencias de infestacion en diversos vertebrados. *Revista Iberica de Parasitologia* **26**, 239–288.
- Hall, M.C. (1937). Studies on trichinosis. IV. The role of the garbage-fed hog in the production of human trichinosis. *Public Health Reports* **52**, 873–886.
- Hanbury, R.D., Doby, P.B., Miller, H.O. and Murrell, K.D. (1986). Trichinosis in a herd of swine: cannibalism as a major mode of transmission. *Journal of American Veterinary Medical Association* **188**, 1155–1159.
- Haydon, D.T., Cleaveland, S., Taylor, L.H. and Laurenson, M.K. (2002). Identifying reservoirs of infection: a conceptual and practical challenge. *Emerging Infectious Diseases* **8**, 1468–1473.
- Heper, Y., Yilmaztepe, F., Komitova, R., Akalin, H., Vutova, K. and Helvacı, S. (2005). A trichinosis outbreak caused by wild boar meat in Turkey. *Parasite* **12**, 191–192.
- Holliman, R.B. and Meade, B.J. (1980). Native trichinosis in wild rodents in Henrico County, Virginia. *Journal Wildlife Diseases* **16**, 205–207.
- Hulebak, K.L. (1980). Mechanical transmission of larval *Trichinella* by Arctic crustacea. *Canadian Journal of Zoology* **58**, 1388–1390.
- Hurniková, Z., Dubinský, P., Mukaratirwa, S., Foggin, F.C. and Kapel, C.M.O. (2004). Infectivity and temperature tolerance of non-encapsulating *Trichinella zimbabwensis* in experimentally infected red foxes (*Vulpes vulpes*). *Helminthologia* **41**, 189–192.
- Hurniková, Z., Šnábel, V., Pozio, E., Reiterová, K., Hrčková, G., Halášová, D. and Dubinský, P. (2005). Domestic focus of *Trichinella pseudospiralis* in the Slovak Republic. *Veterinary Parasitology* **128**, 91–98.
- Imperato, P.J., Harvey, R.P., Shookhoff, H.B. and Chaves, A.D. (1974). Trichinosis among Thais living in New York City. *Journal of American Medical Association* **227**, 526–529.
- International Commission on Trichinellosis. www.med.unipi.it/ict/welcome.htm
- International Trichinella Reference Centre. www.iss.it/site/Trichinella/index.asp
- Jongwutiwes, S., Chantachum, N., Kraivichian, P., Siriwasatien, P., Putaporn-tip, C., Tamburrini, A., La Rosa, G., Sreesunpasirikul, C., Yingyoud, P.

- and Pozio, E. (1998). First outbreak of human trichinellosis caused by *Trichinella pseudospiralis*. *Clinical Infectious Diseases* **26**, 111–115.
- Kapel, C.M.O. (2000). Host diversity and biological characteristics of the *Trichinella* genotypes and their effect on transmission. *Veterinary Parasitology* **93**, 263–278.
- Kapel, C.M.O. (2001). Sylvatic and domestic *Trichinella* spp. in wild boars: infectivity, muscle larvae distribution, and antibody response. *Journal of Parasitology* **87**, 309–314.
- Kapel, C.M.O. (2005). Changes in the EU legislation on *Trichinella* inspection—new challenges in the epidemiology. *Veterinary Parasitology* **132**, 189–194.
- Kapel, C.M.O. and Gamble, H.R. (2000). Infectivity, persistence, and antibody response to domestic and sylvatic *Trichinella* spp. in experimentally infected pigs. *International Journal for Parasitology* **30**, 215–221.
- Kazura, J.W. (1982). Immunology of *Trichinella spiralis* infection. In: *Immunity to Parasitic Infections* (S. Cohen and K.S. Warren, eds), pp. 654–675. Oxford: Blackwell Scientific Publishers.
- Kefenie, H. and Bero, G. (1992). Trichinosis from wild boar meat in Gojjam, north-west Ethiopia. *Tropical and Geographical Medicine* **44**, 278–280.
- Kefenie, H., Wolde, H. and Abuherpo, M. (1988). Trichinosis from wild boar meat in Arsi, central Ethiopia. *Ethiopian Medical Journal* **26**, 97–100.
- Khamboonruang, C. (1991). The present status of trichinellosis in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **22**, 312–315.
- Klassen, G.R., Thiessen, J.P. and Dick, T.A. (1986). Strain-specific 1.7 kilobase-repetitive deoxyribonucleic acid sequence family in *Trichinella spiralis*. *Molecular and Biochemical Parasitology* **21**, 227–233.
- Kociecka, W., van Knapen, F. and Ruitenbergh, E.J. (1981). *Trichinella pseudospiralis* and *T. spiralis* infections in monkeys. I. Parasitological aspects. In: *Trichinellosis* (C.W. Kim, E.J. Ruitenbergh and J.S. Teppema, eds), pp. 199–203. Chertsey, England: Reedbooks.
- Kozar, Z. (1970). Trichinosis in Europe. In: *Trichinosis in Man and Animals* (S.E. Gould, ed.), pp. 423–436. Springfield, IL: Charles C Thomas Publisher.
- Kozar, Z. and Kozar, M. (1965). A comparison of the infectivity and pathogenicity of *Trichinella spiralis* strains from Poland and Kenya. *Journal of Helminthology* **39**, 19–34.
- Kruger, S.P., Collins, M.H., van Niekerk, J.W., McCully, R.M. and Basson, P.A. (1969). Experimental observations on the South Africa strain of *T. spiralis*. *Wiadomosci Parazytologiczne* **15**, 546–554.
- Kumar, V., Pozio, E., De Borchgrave, J., Mortelmans, J. and De Meurichy, W. (1990). Characterization of a *Trichinella* isolate from polar bear. *Annales de la Société Belge de Médecine Tropicale* **70**, 131–135.

- Kurup, A., Yew, W.S., San, L.M., Ang, B., Lim, S. and Tai, G.K. (2000). Outbreak of suspected trichinosis among travellers returning from a neighbouring island. *Journal of Travel Medicine* **7**, 189–193.
- Lanoire, Duchier and Friez. (1963). Onze observations de trichinose en Algérie. *Gazette Hopitale Paris* **135**, 305–310.
- La Rosa, G., Marucci, G. and Pozio, E. (2003a). Biochemical analysis of encapsulated and non-encapsulated species of *Trichinella* (Nematoda, Trichinellidae) from cold- and warm-blooded animals reveals a high genetic divergence in the genus. *Parasitology Research* **91**, 462–466.
- La Rosa, G., Marucci, G., Zarlenga, D.S., Casulli, A., Zarnke, R.L. and Pozio, E. (2003b). Molecular identification of natural hybrids between *Trichinella nativa* and *Trichinella* T6 provides evidence of gene flow and ongoing genetic divergence. *International Journal for Parasitology* **33**, 209–216.
- La Rosa, G., Marucci, G., Zarlenga, D.S. and Pozio, E. (2001). *Trichinella pseudospiralis* populations of the Palearctic region and their relationship with populations of the Nearctic and Australian regions. *International Journal for Parasitology* **31**, 297–305.
- La Rosa, G. and Pozio, E. (2000). Molecular investigation of African isolates of *Trichinella* reveals genetic polymorphism in *Trichinella nelsoni*. *International Journal for Parasitology* **30**, 663–667.
- La Rosa, G., Pozio, E., Rossi, P. and Murrell, K.D. (1992). Allozyme analysis of *Trichinella* isolates from various host species and geographic regions. *Journal of Parasitology* **78**, 641–646.
- La Rosa, G., Tasciotti, L. and Pozio, E. (1994). DNA repetitive probes for the characterization and identification of *Trichinella* parasites. In: *Trichinellosis* (C.W. Campbell, E. Pozio and F. Bruschi, eds), pp. 89–94. Rome, Italy: Istituto Superiore di Sanità Press.
- Larsh, J.E., Jr. (1975). Allergic inflammation as a hypothesis for the expulsion of worms from tissues: a review. *Experimental Parasitology* **37**, 251–266.
- Leiby, D.A., Duffy, C.H., Murrell, K.D. and Schad, G.A. (1990). *Trichinella spiralis* in an agricultural ecosystem: transmission in the rat population. *Journal of Parasitology* **76**, 360–364.
- Leiby, D.A., Schad, G.A., Duffy, C.H. and Murrell, K.D. (1988). *Trichinella spiralis* in an agricultural ecosystem. III. Epidemiological investigations of *Trichinella spiralis* in resident wild and feral animals. *Journal of Wildlife Diseases* **24**, 606–609.
- Liberona, H.E. and MacDiarmid, S.C. (1988). *Trichinella spiralis* in New Zealand. *Surveillance* **15**, 17–18.
- Lichtenfels, J.R., Murrell, K.D. and Pilitt, P.A. (1983). Comparison of three subspecies of *Trichinella spiralis* by scanning electron microscopy. *Journal of Parasitology* **69**, 1131–1140.

- Lindsay, D.S., Zarlenga, D.S., Gamble, H.R., Al-Yaman, F., Smith, P.C. and Blagburn, B.L. (1995). Isolation and characterization of *Trichinella pseudospiralis* Garkavi, 1972 from a black vulture (*Coragyps atratus*). *Journal of Parasitology* **81**, 920–923.
- Liu, M. and Boireau, P. (2002). Trichinellosis in China: epidemiology and control. *Trends in Parasitology* **18**, 553–556.
- MacDonald, D.W. (1980). *Rabies and wildlife: a biologist's perspective*. Oxford, UK: Oxford University Press.
- MacLean, J.D., Viallet, J., Law, C. and Staudt, M. (1989). Trichinosis in the Canadian Arctic: report of five outbreaks and a new clinical syndrome. *Journal of Infectious Diseases* **60**, 513–520.
- Madsen, H. (1974). The principles of the epidemiology of trichinellosis with a new view on the life cycle. In: *Trichinellosis* (C.W. Kim, ed.), pp. 615–638. New York: Intext Educational Publishers.
- Malakauskas, A. (2002). *Molecular epidemiology of trichinellosis in Lithuania and biological characteristics of Trichinella genotypes in rat*. Ph.D. thesis, Copenhagen, Denmark: The Royal Veterinary and Agricultural University.
- Malakauskas, A. and Kapel, C.M. (2003). Tolerance to low temperatures of domestic and sylvatic *Trichinella* spp. in rat muscle tissue. *Journal of Parasitology* **89**, 744–748.
- Mantovani, A., Filippini, I. and Bergomi, S. (1980). Findings on an epidemic of human trichinelliasis verified in Italy. *Parassitologia* **22**, 107–134 [In Italian].
- Margolis, H.S., Mittlehaugh, J.P. and Burgess, R.D. (1979). Arctic trichinosis: two Alaskan outbreaks from walrus meat. *Journal of Infectious Diseases* **139**, 102–105.
- Maroli, M. and Pozio, E. (2000). Influence of temperature on the survival and infectivity of *Trichinella spiralis* larvae in *Sarcophaga argyrostoma* (Diptera, Sarcophagidae) maggots. *Journal of Parasitology* **86**, 633–634.
- Marti, H.P. and Murrell, K.D. (1986). *Trichinella spiralis*: anti-fecundity and anti-newborn larvae immunity in swine. *Experimental Parasitology* **62**, 370–375.
- Marva, E., Markovics, A., Gdelevich, M., Asor, N., Sadik, C. and Leventhal, A. (2005). Trichinellosis outbreak. *Emerging Infectious Diseases* **11**, 1979–1981.
- Mason, P.C. (1978). *Trichinella spiralis* in New Zealand. *New Zealand Veterinary Journal* **26**, 215–216.
- Mayer, J.J. and Brisbin, Jr. (1991). *Wild pigs in the United States*. Athens: The University Press.
- McAuley, J.B., Michelson, M.K., Hightower, A.W., Engeran, S., Wintermeyer, L.A. and Schantz, P.M. (1992). A trichinosis outbreak among Southeast Asian refugees. *American Journal of Epidemiology* **135**, 1404–1410.
- McAuley, J.B., Michelson, M.K. and Schantz, P.M. (1991). *Trichinella* infection in travelers. *Journal of Infectious Diseases* **164**, 1013–1016.

- McCoy, O.R. (1932). Experimental trichiniasis infections in monkeys. *Proceedings of the Society of Biological Medicine* **30**, 85–86.
- Mémin, Y., Pernod, J., Papillaud, J. and Morelle, M. (1968). Aspects actuels du diagnostic et du traitement de la trichinose humaine (a propos d'une observation récente recueillie en France). *Société Médicale Hôpitaux Paris* **119**, 113–123.
- Merkushev, A.V. (1955). Concerning the rotation of *Trichinella* invasions in nature and their natural foci. *Medicinskaja Parazitologia* **2**, 125–130 [in Russian].
- Merkushev, A.V. (1970). Trichinosis in the Union of Soviet Socialist Republics. In: *Trichinosis in Man and Animals* (S.E. Gould, ed.), pp. 449–456. Springfield, IL: Charles C. Thomas Publisher.
- Michel, P., Zurlinden, A., Charvillat, L., Levenecq, P., Janin, G., Bayle, A. and Mojon, M. (1986). Cinq nouveaux cas de trichinose. *Presse Medicale* **15**, 2073–2074.
- Mikhail, E.M., Mansour, N.S. and Awadalla, H.N. (1994). Identification of *Trichinella* isolates from naturally infected stray dogs in Egypt. *Journal of Parasitology* **80**, 151–154.
- Mikkonen, T., Valkama, J., Wihlman, H. and Sukura, A. (2005). Spatial variation of *Trichinella* prevalence in rats in Finnish waste disposal sites. *Journal of Parasitology* **91**, 210–213.
- Miller, I., Jarvis, T. & Pozio, E. (2006). Epidemiological investigations on *Trichinella* infections in farmed fur animals of Estonia. *Veterinary Parasitology* (in press).
- Minchella, D.J., Branstetter, B.A. and Kazacos, K.R. (1989). Molecular characterization of sylvatic isolates of *Trichinella spiralis*. *Journal of Parasitology* **75**, 388–392.
- Møller, L.N., Petersen, E., Kapel, C.M.O., Melbye, M. and Koch, A. (2005). Outbreak of trichinellosis associated with consumption of game meat in West Greenland. *Veterinary Parasitology* **132**, 131–136.
- Moretti, A., Piergili Fioretti, D., Pasquali, P., Mechelli, L., Rossodivita, M.E. and Polidori, G.A. (1997). Experimental infection of fish with *Trichinella britovi*: biological evaluations. In: *Trichinellosis* (G. Ortega-Pierres, H.R. Gamble, F. van Knapen and D. Wakelin, eds), pp. 135–142. Mexico City: Centro de Investigación y Estudios Avanzados IPN.
- Mukaratirwa, S., Dzoma, B.M., Mantenga, E., Ruziwa, S.D., Sacchi, L. and Pozio, E. (2005). Experimental infections of baboons (*Papio* spp.) and monkeys (*Cercopithecus aethiops*) with *Trichinella zimbabwensis* and successful treatment with Ivermectin. *Proceedings of the 20th International Conference of the World Association for the Advancement of Veterinary Parasitology*, 120.
- Mukaratirwa, S. and Foggin, C.M. (1999). Infectivity of *Trichinella* sp. isolated from *Crocodylus niloticus* to the indigenous Zimbabwean pig (Mukota). *International Journal for Parasitology* **29**, 1129–1131.

- Mukaratirwa, S., Magwedere, K., Matenga, E. and Foggin, C.M. (2001). Transmission studies on *Trichinella* species isolated from *Crocodylus niloticus* and efficacy of fenbendazole and levamisole against muscle L1 stages in Balb C mice. *Onderstepoort Journal Veterinary Research* **68**, 21–25.
- Mukaratirwa, S., Nkulungo, E., Matenga, E. and Bhebhe, E. (2003). Effect of host age in the distribution of adult *Trichinella zimbabwensis* in the small intestines of golden hamsters (*Mesocricetus auratus*) and Balb C mice. *Onderstepoort Journal Veterinary Research* **70**, 169–173.
- Murrell, K.D. (1985). *Trichinella spiralis*: acquired immunity in swine. *Experimental Parasitology* **59**, 347–354.
- Murrell, K.D. (1994). Beef as source of trichinellosis. *Parasitology Today* **10**, 434.
- Murrell, K.D. and Pozio, E. (2000). Trichinellosis: the zoonosis that won't go quietly. *International Journal for Parasitology* **30**, 1339–1349.
- Murrell, K.D., Marti, H. and Gamble, H.R. (1987b). Recent progress in the vaccines against animal parasites. In: *Vaccines: New Concepts and Developments* (H. Kohler and P.T. Lo Verde, eds), pp. 403–412. New York: Longman Scientific and Technical Publications.
- Murrell, K.D., Lieby, D.A., Duffy, C. and Schad, G.A. (1985). Susceptibility of domestic swine to wild animal isolates of *Trichinella spiralis*. In: *ICT6 Trichinellosis* (C.W. Kim, ed.), pp. 301–305. Albany: The State University of New York Press.
- Murrell, K.D., Stringfellow, F., Dame, J.B., Leiby, D.A., Duffy, C. and Schad, G.A. (1987a). *Trichinella spiralis* in an agricultural ecosystem. II. Evidence for natural transmission of *Trichinella spiralis* from domestic swine to wildlife. *Journal of Parasitology* **73**, 103–109.
- Murrell, K.D., Lichtenfels, R.J., Zarlenga, D.S. and Pozio, E. (2000). The systematics of *Trichinella* with a key to species. *Veterinary Parasitology* **93**, 293–307.
- Murrell, K.D., Djordjevic, M., Cuperlovic, K., Sofronic, Lj., Savic, M., Djordjevic, M. and Damjanovic, S. (2004). Epidemiology of *Trichinella* infection in the horse: the risk from animal product feeding practices. *Veterinary Parasitology* **123**, 223–233.
- Mydnyski, L.J. and Dick, T.A. (1985). The use of enzyme polymorphisms to identify genetic differences in the genus *Trichinella*. *Journal of Parasitology* **71**, 671–677.
- Nadzhimiddinov, T.K., Dzhaifarov, A.D. and Tsipkina, S.V. (1965). Mass outbreak of trichinelliasis from meat of wild boar in the Uzbek SSR. *Soviet Medicine* **28**, 136–138 [in Russian].
- Nagano, I., Wu, Z., Matsuo, A., Pozio, E. and Takahashi, Y. (1999). Identification of *Trichinella* genotypes by polymerase chain reaction—restriction fragment length polymorphism of mitochondrial cytochrome c oxidase subunit I gene. *International Journal for Parasitology* **29**, 1113–1120.

- Nakamura, T., Miura, T., Nakaoka, T., Nagano, I., Takahashi, Y. and Iwamoto, A. (2003). A case of trichinellosis with spontaneous remission. *Kansenshogaku Zasshi* **77**, 839–843 [in Japanese].
- Nelson, G.S. (1970). Trichinosis in Africa. In: *Trichinosis in Man and Animals* (S.E. Gould, ed.), pp. 473–492. Springfield, IL: Charles C. Thomas Publisher.
- Nelson, G.S. (1988). More than a hundred years of parasitic zoonoses: with special reference to trichinosis and hydatid disease. *Journal of Comparative Pathology* **98**, 135–153.
- Nelson, G.S. and Mukundi, J. (1963). A strain of *Trichinella spiralis* from Kenya of low infectivity to rats and domestic pigs. *Journal of Helminthology* **37**, 329–338.
- Nelson, G.S., Blackie, E.J. and Mukundi, J. (1966). Comparative studies on geographical strains of *Trichinella spiralis*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **60**, 471–480.
- Nelson, M., Wright, T.L., Pierce, A. and Krogwold, R.A. (2003). A common-source outbreak of trichinosis from consumption of bear meat. *Journal of Environmental Health* **65**, 16–19, 24.
- Nezri, M., Ruer, J., De Bruyne, A., Cohen-Valensi, R., Pozio, E. and Dupouy-Camat, J. (2006). Première observation d'un cas de humain de trichinellose à *Trichinella britovi* en Algérie après consommation de viande de chacal (*Canis aureus*). *Bulletin de la Société de Pathologie Exotique* **99**, 94–95.
- Niphadkar, S.M. (1973). *Trichinella spiralis* (Owen, 1835) in *Bandicota bengalensis* (Gray) in Bombay. *Current Science* **42**, 135–136.
- Niquet, G., Vernes, A., Havet, S., Dupont, T.H. and Gobert, P. (1979). La trichinose (à propos d'une observation). *Lille Médicale* **24**, 376–379.
- Nöckler, K., Reckinger, S. and Pozio, E. (2006). *Trichinella spiralis* and *Trichinella pseudospiralis* mixed infection in a wild boar (*Sus scrofa*) of Germany. *Veterinary Parasitology* **137**, 364–368.
- Nozais, J.P., Mannevy, V. and Danis, M. (1996). Deux cas de trichinose après ingestion de viande d'ours blanc au Groenland. *Medecine Malattie Infectives* **26**, 732–733.
- Obendorf, D.L. and Clarke, K.P. (1992). *Trichinella pseudospiralis* infections in free-living Tasmanian birds. *Journal of Helminthological Society of Washington* **59**, 144–147.
- Obendorf, D.L., Handler, J.H., Mason, R.W., Clarke, K.P., Forman, A.J., Hooper, P.T., Smith, S.J. and Holdsworth, M. (1990). *Trichinella pseudospiralis* infection in Tasmanian wildlife. *Australian Veterinary Journal* **67**, 108–110.
- Oivanen, L., Kapel, C.M.O., Pozio, E., La Rosa, G., Mikkonen, T. and Sukura, A. (2002). Associations between *Trichinella* species and host species in Finland. *Journal of Parasitology* **88**, 84–88.
- Olaison, L. and Ljungstrom, I. (1992). An outbreak of trichinosis in Lebanon. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 658–660.

- Olteanu, G. (2001). Trichinellosis in Romania: a short review over the past twenty years. *Parasite* **8**(S2), S98–S99.
- Ortega Pierres, M.G., Arriaga, C. and Yepez-Mulia, L. (2000). Epidemiology of trichinellosis in Mexico, Central and South America. *Veterinary Parasitology* **93**, 201–225.
- Owen, R. (1835). Description of a microscopic entozoon infesting the muscles of the human body. *Transactions of the Zoological Society of London* **1**, 315–324.
- Owen, I.L., Gomez Morales, M.A., Pezzotti, P. and Pozio, E. (2005). *Trichinella* infection in a hunting population of Papua New Guinea suggests an ancient relationship of *Trichinella* with human beings. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**, 618–624.
- Owen, I.L., Pozio, E., Tamburrini, A., Danaya, R.T., Bruschi, F. and Gomez Morales, M.A. (2001). Focus of human trichinellosis in Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* **65**, 553–557.
- Owen, I.L., Sims, L.D., Wigglesworth, M.C. and Puana, I. (2000). Trichinellosis in Papua New Guinea. *Australian Veterinary Journal* **78**, 698–701.
- Ozdemir, D., Ozkan, H., Akkoc, N., Onen, F., Gurler, O., Sari, I., Akar, S., Birlik, M., Kargi, A., Ozer, E. and Pozio, E. (2005). Acute trichinellosis in children compared with adults. *Pediatric Infectious Disease Journal* **24**, 897–900.
- Paterson, K., Black, A. and Reichel, M.P. (1997). Porcine trichinosis diagnosed in the Rotorua area. *Surveillance* **24**, 6–8.
- Pedersen, S. and Murrell, K.D. (2001). Whipworm-nutrition interaction. *Trends in Parasitology* **17**, 470.
- Pedersen, S., Saeed, I., Michaelsen, K.F., Friis, H. and Murrell, K.D. (2002). Impact of protein energy malnutrition on *Trichuris suis* infection in pigs concomitantly infected with *Ascaris suum*. *Parasitology* **124**, 561–568.
- Peklo, G.N., Skarednov, N.I., Stepanova, T.F. and Filatov, V.G. (2002). Serological assay in the assessment of the trichinosis epidemiological situation in the Tiumen' region. *Meditsinskaja parazitologija i parazytarnie bolezni*, **1**, 22–24 (Jan–Mar) [in Russian].
- Perdomo Gonzalez, G., Perez Nellar, J. and Vega Garcia, E. (1986). Trichinosis from wild boar meat in Ethiopia: a case report. *Ethiopian Medical Journal* **24**, 93–96.
- Perevertseva, E.V. (1966). On the question of strains of *Trichinella*. *Wiadomosci Parazytologiczne* **12**, 531–541 [in Russian].
- Pérez-Martin, J., Serrano, F.J., Reina, D., Mora, J.A. and Navarrete, I. (2000). Sylvatic trichinellosis in southwestern Spain. *Journal of Wildlife Diseases* **36**, 531–534.
- Pozio, E. (1987). Isoenzymatic typing of 23 *Trichinella* isolates. *Tropical Medicine and Parasitology* **38**, 111–116.

- Pozio, E. (1998). Trichinellosis in the European Union: Epidemiology, ecology and economic impact. *Parasitology Today* **14**, 35–38.
- Pozio, E. (2000). The domestic, synanthropic and sylvatic cycles of *Trichinella* and the flow among them. *Veterinary Parasitology* **93**, 241–262.
- Pozio, E. (2001a). New patterns of *Trichinella* infection. *Veterinary Parasitology* **98**, 133–148.
- Pozio, E. (2001b). Taxonomy of *Trichinella* and the epidemiology of the infection in the south-east Asia and Australian regions. *South East Asian Journal of Tropical Medicine and Public Health* **32**(S2), 129–132.
- Pozio, E. (2005). The broad spectrum of *Trichinella* hosts: from cold- to warm-blooded animals. *Veterinary Parasitology* **132**, 3–11.
- Pozio, E. and Kapel, C.M.O. (1999). *Trichinella nativa* in sylvatic wild boars. *Journal of Helminthology* **73**, 87–89.
- Pozio, E. and La Rosa, G. (1998). Identification of the likely etiological agent of human trichinellosis in Sicily (Italy) between 1933 and 1946. *American Journal of Tropical Medicine and Hygiene* **59**, 906–907.
- Pozio, E. and La Rosa, G. (2000). *Trichinella murrelli* n. sp: etiological agent of sylvatic trichinellosis in temperate areas of North America. *Journal of Parasitology* **86**, 134–139.
- Pozio, E. and La Rosa, G. (2003). PCR-derived methods for the identification of *Trichinella* parasites from animal and human samples. *Methods in Molecular Biology* **216**, 299–309.
- Pozio, E. and La Rosa, G. (2005). Evaluation of the infectivity of *Trichinella papuae* and *Trichinella zimbabwensis* for equatorial freshwater fishes. *Veterinary Parasitology* **132**, 113–114.
- Pozio, E. and Marucci, G. (2003). *Trichinella*-infected pork products: a dangerous gift. *Trends in Parasitology* **19**, 338.
- Pozio, E. and Zarlenga, D.S. (2005). Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. *International Journal for Parasitology* **35**, 1191–1204.
- Pozio, E., La Rosa, G. and Rossi, P. (1989). *Trichinella* Reference Centre. *Parasitology Today* **5**, 169–170.
- Pozio, E., La Rosa, G., Murrell, K.D. and Lichtenfels, J.R. (1992a). Taxonomic revision of the genus *Trichinella*. *Journal of Parasitology* **78**, 654–659.
- Pozio, E., La Rosa, G., Rossi, P. and Murrell, K.D. (1992b). Biological characterizations of *Trichinella* isolates from various host species and geographic regions. *Journal of Parasitology* **78**, 647–653.
- Pozio, E., Shaikenov, B., La Rosa, G. and Obendorf, D.L. (1992c). Allozymic and biological characters of *Trichinella pseudospiralis* isolates from free-ranging animals. *Journal of Parasitology* **78**, 1087–1090.
- Pozio, E., Varese, P., Gomez Morales, M.A., Croppo, G.P., Pelliccia, D. and Bruschi, F. (1993). Comparison of human trichinellosis caused by *Trichinella spiralis* and by *Trichinella britovi*. *American Journal of Tropical Medicine and Hygiene* **48**, 568–575.

- Pozio, E., La Rosa, G. and Amati, M. (1994a). Factors influencing the resistance of *Trichinella* muscle larvae to freezing. In: *Trichinellosis* (W.C. Campbell, F. Bruschi and E. Pozio, eds), pp. 173–178. Rome, Italy: Istituto Superiore di Sanità Press.
- Pozio, E., Verster, A., Braack, L., De Meneghi, D. and La Rosa, G. (1994b). Trichinellosis south of the Sahara. In: *Trichinellosis* (C.W. Campbell, E. Pozio and F. Bruschi, eds), pp. 527–532. Rome, Italy: Istituto Superiore di Sanità Press.
- Pozio, E., Bandi, C., La Rosa, G., Jarvis, T., Miller, I. and Kapel, C.M. (1995). Concurrent Infection with Sibling *Trichinella* Species in a Natural Host. *International Journal for Parasitology* **25**, 1247–1250.
- Pozio, E., La Rosa, G., Yamaguchi, T. and Saito, S. (1996a). *Trichinella britovi* from Japan. *Journal of Parasitology* **82**, 847–849.
- Pozio, E., La Rosa, G., Serrano, F.J., Barrat, J. and Rossi, L. (1996b). Environmental and human influence on the ecology of *Trichinella spiralis* and *Trichinella britovi* in Western Europe. *Parasitology* **113**, 527–533.
- Pozio, E., Serrano, F.J., La Rosa, G., Reina, D., Perez-Martin, E. and Navarrete, I. (1997a). Evidence of potential gene flow in *Trichinella spiralis* and in *Trichinella britovi* in Nature. *Journal of Parasitology* **83**, 163–166.
- Pozio, E., De Meneghi, D., Roelke-Parker, M.E. and La Rosa, G. (1997b). *Trichinella nelsoni* in carnivores from the Serengeti ecosystem, Tanzania. *Journal of Parasitology* **83**, 1195–1198.
- Pozio, E., Miller, I., Jarvis, T., Kapel, C.M.O. and La Rosa, G. (1998a). Distribution of sylvatic species of *Trichinella* in Estonia according to climate zones. *Journal of Parasitology* **84**, 193–195.
- Pozio, E., Sacchini, D., Boni, P., Tamburini, A., Alberici, F. and Paterlini, F. (1998b). Human outbreak of trichinellosis associated with the consumption of horse-meat in Italy. *Eurosurveillance* **3**, 85–86.
- Pozio, E., Owen, I.L., La Rosa, G., Sacchi, L., Rossi, P. and Corona, S. (1999a). *Trichinella papuae* n. sp. (Nematoda), a new non-encapsulated species from domestic and sylvatic swine of Papua New Guinea. *International Journal for Parasitology* **29**, 1825–1839.
- Pozio, E., Goffredo, M., Fico, R. and La Rosa, G. (1999b). *Trichinella pseudospiralis* in sedentary night-birds of prey from Central Italy. *Journal of Parasitology* **85**, 759–761.
- Pozio, E., Tamburrini, A. and La Rosa, G. (2001a). Horse trichinellosis, an unresolved puzzle. *Parasite* **8**(2S), S263–S265.
- Pozio, E., La Rosa, G., D'Ancona, F., Amati, M., Mancini Barbieri, F. and De Giacomo, M. (2001b). Twelve years of activity of the international *Trichinella* reference centre. *Parasite* **8**(S2), S44–S46.
- Pozio, E., Zarlenga, D.S. and La Rosa, G. (2001c). The detection of encapsulated and non-encapsulated species of *Trichinella* suggests the existence of two evolutive lines in the genus. *Parasite* **8**(S2), S27–S29.

- Pozio, E., La Rosa, G. and Gomez Morales, M.A. (2001d). Epidemiology of human and animal trichinellosis in Italy since its discovery in 1887. *Parasite* **8**(S2), S106–S108.
- Pozio, E., Pence, D.B., La Rosa, G., Casulli, A. and Henke, S.E. (2001e). *Trichinella* infection in the southwestern United States. *Journal of Parasitology* **87**, 1208–1210.
- Pozio, E., Casulli, A., Bologov, V.V., Marucci, G. and La Rosa, G. (2001f). Hunting practices increase the prevalence of *Trichinella* infection in wolves from European Russia. *Journal of Parasitology* **87**, 1498–1501.
- Pozio, E., Sacchini, D., Sacchi, L., Tamburrini, A. and Alberici, F. (2001g). Failure of mebendazole in treating *Trichinella spiralis* infection in humans at the stage of encapsulating larvae. *Clinical Infectious Diseases* **32**, 638–642.
- Pozio, E., Foggin, C.M., Marucci, G., La Rosa, G., Sacchi, L., Corona, S., Rossi, P. and Mukaratirwa, S. (2002). *Trichinella zimbabwensis* n. sp. (Nematoda), a new non-encapsulated species from crocodiles (*Crocodylus niloticus*) in Zimbabwe also infecting mammals. *International Journal for Parasitology* **32**, 1787–1799.
- Pozio, E., Gomez Morales, M.A. and Dupouy Camet, J. (2003). Clinical aspects, diagnosis and treatment of trichinellosis. *Expert Review of Anti-infective Therapy* **1**, 471–482.
- Pozio, E., Marucci, G., Casulli, A., Sacchi, L., Mukaratirwa, S., Foggin, C.M. and La Rosa, G. (2004a). *Trichinella papuae* and *Trichinella zimbabwensis* induce infection in experimentally infected varans, caimans, pythons and turtles. *Parasitology* **128**, 333–342.
- Pozio, E., Christensson, D., Steen, M., Marucci, G., La Rosa, G., Brojer, C., Morner, T., Uhlhorn, H., Agren, E. and Hall, M. (2004b). *Trichinella pseudospiralis* foci in Sweden. *Veterinary Parasitology* **125**, 335–342.
- Pozio, E., Owen, I.L., Marucci, G. and La Rosa, G. (2005a). Inappropriate feeding practice favors the transmission of *Trichinella papuae* from wild pigs to saltwater crocodiles in Papua New Guinea. *Veterinary Parasitology* **127**, 245–251.
- Pozio, E., Pagani, P., Marucci, G., Zarlenga, D.S., Hoberg, E.P., De Meneghi, D., La Rosa, G. and Rossi, L. (2005b). *Trichinella britovi* etiological agent of sylvatic trichinellosis in the Republic of Guinea (West Africa) and a re-evaluation of geographical distribution for encapsulated species in Africa. *International Journal for Parasitology* **35**, 955–960.
- Pozio, E., Mesina, P., Sechi, F., Pira, M., Liciardi, M., Cossu, P., Marucci, G., Grippa, G., and Firinu, A. (2006). Human outbreak of trichinellosis in the Mediterranean island of Sardinia, Italy. *Veterinary Parasitology* (in press).
- ProMed mail. (2004). Trichinellosis, dog meat, human, Russia (Buryatiya), archive No. 20050407.10.
- ProMed mail. (2005). Trichinellosis, badger, Novosibirsk region, Russia, archive No. 20050204.

- Proulx, J.F., MacLean, J.D., Gyorkos, T.W., Leclair, D., Richter, A.K., Serhir, B., Forbes, L. and Gajadhar, A.A. (2002). Novel prevention program for trichinellosis in Inuit communities. *Clinical Infectious Diseases* **34**, 1508–1514.
- Pyburn, D.G., Gamble, H.R., Wagstrom, E.A., Anderson, L.A.Q. and Miller, L.E. (2005). Trichinae certification in the United states pork industry. *Veterinary Parasitology* **132**, 179–183.
- Rafter, P., Marucci, G., Brangan, P. and Pozio, E. (2005). Rediscovery of *Trichinella spiralis* in red foxes (*Vulpes vulpes*) in Ireland after thirty years of oblivion. *Journal of Infectious* **50**, 61–65.
- Railliet, A. (1896). Quelques rectifications a la nomenclature des parasites. *Recueil de Médecine Vétérinaire* **3**, 157–161.
- Ranque, S., Faugère, B., Pozio, E., La Rosa, G., Tamburrini, A., Pellissier, J.F. and Brouqui, P. (2000). *Trichinella pseudospiralis* outbreak in France. *Emerging Infectious Diseases* **6**, 543–547.
- Rausch, R.L. (1970). Trichinosis in the Arctic. In: *Trichinosis in man and animals* (S.E. Gould, ed.), pp. 348–373. Springfield, IL: Charles C. Thomas Publisher.
- Rausch, R.L., Babero, B.B., Rausch, R.V. and Schiller, E.L. (1956). Studies on the helminth fauna of Alaska. XXVII. The occurrence of larvae of *Trichinella spiralis* in Alaskan mammals. *Journal of Parasitology* **42**, 259–271.
- Reina, D., Munoz-Ojeda, M.C., Serrano, F., Molina, J.M. and Navarrete, I. (1996). Experimental trichinellosis in goats. *Veterinary Parasitology* **62**, 125–132.
- Ribicich, M., Gamble, H.R., Rosa, A., Bolpe, J. and Franco, A. (2005). Trichinellosis in Argentina: an historical review. *Veterinary Parasitology* **132**, 137–142.
- Robinson, H.A. and Olsen, O.W. (1960). The role of rats and mice in the transmission of the porkworm, *Trichinella spiralis* (Owen, 1835) Railliet, 1895. *Journal of Parasitology* **48**, 589–597.
- Romaris, F. and Appleton, J.A. (2001). Invasion of epithelial cells by *Trichinella spiralis*: *in vitro* observations. *Parasite* **8**(S2), S48–S50.
- Rombout, Y.B., Bosch, S. and van Der Giessen, J.W. (2001). Detection and identification of eight *Trichinella* genotypes by reverse line blot hybridization. *Journal of Clinical Microbiology* **39**, 642–646.
- Roy, S.L., Lopez, A.S. and Schantz, P.M. (2003). Trichinellosis surveillance-United States, 1997–2001. *MMWR Surveillance Summaries* **52**, 1–8.
- Sachs, R. (1970). Zur Epidemiologie der Trichinellose in Afrika. *Zeitschrift für Tropenmedizin und Parasitologie* **20**, 117–126.
- Schad, G.A., Leiby, D.A. and Murrell, K.D. (1984). Distribution, prevalence and intensity of *Trichinella spiralis* infection in furbearing mammals of Pennsylvania. *Journal of Parasitology* **70**, 372–377.
- Schad, G.A., Duffy, C.H., Leiby, D.A., Murrell, K.D. and Zirkle, E.W. (1987). *Trichinella spiralis* in an agricultural ecosystem: transmission

- under natural and experimentally modified on-farm conditions. *Journal of Parasitology* **73**, 95–102.
- Schellenberg, R.S., Tan, B.J., Irvine, J.D., Stockdale, D.R., Gajadhar, A.A., Serhir, B., Botha, J., Armstrong, C.A., Woods, S.A., Blondeau, J.M. and McNab, T.L. (2003). An outbreak of trichinellosis due to consumption of bear meat infected with *Trichinella nativa*, in 2 northern Saskatchewan communities. *Journal of Infectious Diseases* **188**, 835–843.
- Schenone, H., del C. Contreras, M., Sandoval, L., Rojas, A., Favi, M., Acunis, M., Munoz, M. and Antman, G. (1994). Present status of epidemiology of trichinellosis in Chile. In: *Trichinellosis* (C.W. Campbell, E. Pozio and F. Bruschi, eds), pp. 545–548. Rome, Italy: Istituto Superiore di Sanità Press.
- Serhir, B., MacLean, J.D., Healey, S. and Forbes, L. (2001). Outbreak of trichinellosis associated with arctic walruses in northern Canada. *Canadian Community Disease Reports* **27**, 31–36.
- Shaikenov, B.S. (1980). Spontaneous infection of birds with *Trichinella pseudospiralis* Garkavi, 1972. *Folia Parasitologica* **27**, 259–271.
- Shaikenov, B.S. (1992). Ecological border of distribution of *Trichinella nativa* Britov and Boev 1972 and *T. nelsoni* Britov and Boev 1972. *Wiadomosci Parazytologiczne* **38**, 85–91.
- Shaikenov, B.S. and Boev, S.N. (1983). Distribution of *Trichinella* species in the old world. *Wiadomosci Parazytologiczne* **29**, 595–608.
- Shiota, T., Arizono, N., Yoshioka, T., Ishikawa, Y., Fujitake, J., Fujii, H., Tatsuoka, Y. and Kim, Y. (1999). Imported trichinellosis with severe myositis—report of a case. *Kansenshogaku Zasshi* **73**, 76–82 [in Japanese].
- Smith, H.J. (1975). An evaluation of low temperature sterilization of trichinae infected pork. *Canadian Journal Comparative Medicine* **39**, 316–320.
- Smith, H.J., Anzengruber, A. and DuPlessis, D.M. (1976). Current status of trichinosis in swine in the Atlantic provinces. *Canadian Veterinary Journal* **17**, 72–75.
- Smith, H.J. and Kay, E.K. (1987). Role of rats in the transmission of *Trichinella spiralis* to swine. *Canadian Veterinary Journal* **28**, 604.
- Smith, H.J., Snowdon, K.E., Finley, G.G. and Laflamme, L.F. (1990). Pathogenesis and serodiagnosis of experimental *Trichinella spiralis spiralis* and *Trichinella spiralis nativa* infections in cattle. *Canadian Journal Veterinary Research* **54**, 355–359.
- Snyder, D.E., Zarlenga, D.S., La Rosa, G. and Pozio, E. (1993). Biochemical, biological, and genetic characterization of a sylvatic isolate of *Trichinella*. *Journal of Parasitology* **79**, 347–352.
- Sohn, W.M., Kim, H.M., Chung, D.I. and Yee, S.T. (2000). The first human case of *Trichinella spiralis* infection in Korea. *Korean Journal of Parasitology* **38**, 111–115.

- Solomon, G.B. and Warner, G.S. (1969). *Trichinella spiralis* in mammals at Mountain Lake, Virginia. *Journal of Parasitology* **55**, 730–732.
- Solomons, N.W. and Scott, E. (1994). Nutritional status of host populations influences parasitic infections. In: *Parasitic and Infectious Diseases: Epidemiology and Ecology* (E. Scott and G. Smith, eds), pp. 101–114. New York: Academic Press.
- Soule, C., Guillou, J.P., Dupouy-Camet, J., Vallet, C. and Pozio, E. (1993). Differentiation of *Trichinella* isolates by polymerase chain reaction. *Parasitology Research* **79**, 461–465.
- Stehr-Green, J.K. and Schantz, P.M. (1986). Trichinosis in Southeast Asian refugees in the United States. *American Journal of Public Health* **76**, 1238–1239.
- Stewart, G.L. (1983). Biochemistry. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 153–172. New York and London: Plenum Press.
- Stewart, G.L. (1989). Biological and immunological characteristics of *Trichinella pseudospiralis*. *Parasitology Today* **5**, 344–349.
- Stewart, G.L., Kennedy, R.R. and Larsen, E. (1990). Infectivity of *Trichinella pseudospiralis* isolated from carrion. *Journal of Parasitology* **76**, 750–751.
- Stojcevic, D., Zivicnjak, T., Marinculic, A., Marucci, G., Andelko, G., Brstilo, M., Pavo, L. and Pozio, E. (2004). The epidemiological investigation of *Trichinella* infection in brown rats (*Rattus norvegicus*) and domestic pigs in Croatia suggests that rats are not a reservoir at the farm level. *Journal of Parasitology* **90**, 666–670.
- Sukhdeo, M.V. and Meerovitch, E. (1977). Comparison of three geographical isolates of *Trichinella*. *Canadian Journal of Zoology* **55**, 2060–2064.
- Suzdaltsev, A.A., Verkhovtsev, V.N., Spiridonov, A.M., Vekhova, E.V., Serbina, V.A. and Alexeyev, O.A. (1999). Trichinosis outbreak after ingestion of barbecued badger. *International Journal of Infectious Diseases* **3**, 216.
- Takahashi, Y., Mingyuan, L. and Waikagul, J. (2000). Epidemiology of trichinellosis in Asia and Pacific Rim. *Veterinary Parasitology* **93**, 227–239.
- Theodoropoulos, G., Kapel, C.M., Webster, P., Saravanos, L., Zaki, J. and Koutsotolis, K. (2000). Infectivity, predilection sites, and freeze tolerance of *Trichinella* spp. in experimentally infected sheep. *Parasitology Research* **86**, 401–405.
- Therizol, M., Levy, R., Coulbois, J., Brochard, C., Berque, A. and Beto-urne, C. (1975). Trichinose aigue. A propos de quelques cas récents importés d’Egypte. *Bulletin de la Société de Pathologie Exotique* **68**, 407–415.
- Thornbury, F.J. (1897). The pathology of trichinosis. Original observations. *University Medical Magazine* **10**, 64–79.

- Tomasovicova, O. (1981). The role of fresh water fish in transfer and maintenance of Trichinellae under natural conditions. *Biologia* **36**, 115–125.
- Turk, M., Kaptan, F., Turker, N., Korkmaz, M., El, S., Ozkaya, D., Ural, S., Vardari, I., Alkan, M.Z., Coskun, N.A., Turker, M. and Pozio, E. (2006). Clinical and laboratory aspects of a trichinellosis outbreak in Izmir, Turkey. *Parasite* **13**, 65–70.
- van der Giessen, J.W.B., Fonville, M., de Vries, A., Briels, I., van Eckerveld, M. and Teunis, P. (2004). Epidemiology of *Trichinella* in wildlife in the Netherlands and the first isolation of *T. pseudospiralis*. *XIth International Conference on Trichinellosis*, abstract No. 58. San Diego, CA, 8–12 August.
- Venturiello, S.M., Ben, G.J., Costantino, S.N., Malmassari, S.L., Nunez, G.G., Veneroni, R.L. and Traversa, M.J. (1998). Diagnosis of porcine trichinellosis: parasitological and immunoserological tests in pigs from endemic areas of Argentina. *Veterinary Parasitology* **74**, 215–228.
- Verdaguer, S., Hiltenbrand, C., Dangoumau, J. and Vaillant, J.P. (1963). A propos d'un cas de trichinose humaine. *Journal de Médecine de Bordeaux et des Sud-Ouest* **11**, 1687–1694.
- Waddell, H. (1969). The search for *Trichinella spiralis* in Australia. *Australian Veterinary Journal* **45**, 207.
- Wakelin, D. and Denham, D.A. (1983). The immune response. In: *Trichinella and Trichinosis* (W.C. Campbell, ed.), pp. 268–308. New York and London: Plenum Press.
- Wakelin, D. and Grecis, R.K. (1997). T cell and cytokine responses to infections with *Trichinella spiralis* in mice. In: *Trichinellosis ICT9* (G. Ortega-Pierres, R. Gamble, F. van Knapen and D. Wakelin, eds), pp. 339–341. D.F. México: Centro de Investigacion y Estudios Avanzados de Instituto Politecnico Nacional México.
- Wang, Z.Q. and Cui, J. (2001). Epidemiology of swine trichinellosis in China. *Parasite* **8**(S2), S67–S70.
- Wang, Z., Cui, J., Li, H., Wu, C. and Mao, F. (1998). Some observations on trichinellosis in China. *Helminthologia* **35**, 27–29.
- Wang, H., Zhang, Y., Lao, W. and Wu, Z. (1995). Restriction fragment length polymorphism (RFLP) analysis of genomic DNA of 5 strains of *Trichinella spiralis* in China. *Chinese Medical Science Journal* **10**, 131–135.
- Webster, P., Malakauskas, A. and Kapel, C.M.O. (2002). Infectivity of *Trichinella papuae* for experimentally infected red foxes (*Vulpes vulpes*). *Veterinary Parasitology* **105**, 215–218.
- Weyermann, D., Worley, D.E. and Seese, F.M. (1993). Survey of *Trichinella nativa* in Alaskan polar bears *Ursus maritimus*. *Helminthologia* **30**, 143–145.
- Wheeldon, E.B., Dick, T.A. and Schultz, T.A. (1983). First report of *Trichinella spiralis* var. *pseudospiralis* in North America. *Journal of Parasitology* **69**, 781–782.

- Worley, D.E., Seesee, F.M., Espinosa, R.H. and Sterner, M.C. (1986). Survival of sylvatic *Trichinella spiralis* isolates in frozen tissue and processed meat products. *Journal of American Veterinary Association* **189**, 1047–1049.
- Worley, D.E., Seesee, F.M., Zarlenga, D.S. and Murrell, K.D. (1994). Attempts to eradicate trichinellosis from a wild boar population in a private game park (USA). In: *Trichinellosis* (C.W. Campbell, E. Pozio and F. Bruschi, eds), pp. 611–616. Rome, Italy: Istituto Superiore di Sanità Press.
- Worley, D.E., Zarlenga, D.S. and Seesee, F.M. (1990). Freezing resistance of a *Trichinella spiralis* *nativa* isolate from a gray wolf, *Canis lupus*, in Montana, with observations on genetic and biological characteristics of the biotype. *Journal of the Helminthological Society of Washington* **57**, 57–60.
- Wu, Z., Nagano, I., Nakada, T. and Takahashi, Y. (2002). Expression of excretory and secretory protein genes of *Trichinella* at muscle stage differs before and after cyst formation. *Parasitology International* **51**, 155–161.
- Wu, Z., Nagano, I., Pozio, E. and Takahashi, Y. (1999). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the identification of *Trichinella* isolates. *Parasitology* **118**, 211–218.
- Wu, Z., Nagano, I. and Takahashi, Y. (1998). The detection of *Trichinella* with polymerase chain reaction (PCR) primers constructed using sequences of random amplified polymorphic DNA (RAPD) or sequences of complementary DNA encoding excretory-secretory (E-S) glycoproteins. *Parasitology* **117**, 173–183.
- Wu, Z., Nakada, T., Nagano, I. and Takahashi, Y. (2000). DNA fingerprints of *Trichinella* as revealed by restriction fragment length polymorphism and single-strand conformational polymorphism (RFLP-SSCP). *Molecular and Cellular Probes* **14**, 291–297.
- Xu, D., Wu, Z., Nagano, I. and Takahashi, Y. (1997). A muscle larva of *Trichinella pseudospiralis* is intracellular, but does not form a typical cyst wall. *Parasitology International* **46**, 1–5.
- Yao, C., Prestwood, A.K. and McGraw, R.A. (1997). *Trichinella spiralis* (T1) and *Trichinella* T5: a comparison using animal infectivity and molecular biology techniques. *Journal of Parasitology* **83**, 88–95.
- Yimam, A.E., Oku, Y., Nonaka, N., Sakai, H., Morishima, Y., Matsuo, K., La Rosa, G., Pozio, E., Yagi, K. and Kamiya, M. (2001). First report of *Trichinella nativa* in red foxes (*Vulpes vulpes schrencki*) from Otaru City, Hokkaido, Japan. *Parasitology International* **50**, 121–127.
- Young, E. and Kruger, S.P. (1967). *Trichinella spiralis* (Owen, 1935) Railliet, 1895. Infestation of wild carnivores and rodents in South Africa. *Journal of the South Africa Veterinary Medical Association* **38**, 441–443.
- Young, E. and Whyte, I.J. (1975). Trichinosis (*Trichinella spiralis* infestations) in wild animals of the Kruger National Park. *Journal of South Africa Veterinary Association* **46**, 233–234.

- Zarlenga, D.S. (1994). The application of repetitive DNA probes in assessing the potential for hybridization between *Trichinella nativa* and *Trichinella spiralis*. In: *Trichinellosis* (W.C. Campbell, E. Pozio and F. Bruschi, eds), pp. 117–122. Rome, Italy: Istituto Superiore di Sanità Press.
- Zarlenga, D.S. (1997). Molecular aspects of speciation in the genus *Trichinella*. In: *Trichinellosis ICT9* (G.M. Ortega-Pierres, R. Gamble, F. van Knapen and D. Wakelin, eds), pp. 43–46. D.F. Mexico: Centro de Investigacion y Estudios Avanzados Instituto Politecnico Nacional México.
- Zarlenga, D.S. and Barta, J.R. (1990). DNA analysis in the diagnosis of infection and in the speciation of nematode parasites. *Revue Scientifique et Technique de l'Office International des Epizooties* **9**, 533–554.
- Zarlenga, D.S., Chute, M.B., Martin, A. and Kapel, C.M. (1999). A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. *International Journal for Parasitology* **29**, 1859–1867.
- Zarlenga, D.S. and Dame, J.B. (1992). The identification and characterization of a break within the large subunit ribosomal RNA of *Trichinella spiralis*: comparison of gap sequences within the genus. *Molecular and Biochemical Parasitology* **51**, 281–290.
- Zarlenga, D.S., Al-Yaman, F., Minchella, D. and La Rosa, G. (1991). A repetitive DNA probe specific for a North American sylvatic genotype of *Trichinella*. *Molecular and Biochemical Parasitology* **48**, 131–138.
- Zarlenga, D.S., Aschenbrenner, R.A. and Lichtenfels, J.R. (1996). Variations in microsatellite sequences provide evidence for population differences and multiple ribosomal gene repeats within *Trichinella pseudospiralis*. *Journal of Parasitology* **82**, 534–538.
- Zarlenga, D.S., La Rosa, G., Pozio, E. and Rosenthal, B. (2004). Identification and classification within the genus *Trichinella*, with special emphasis on non-encapsulated species. *Veterinary Parasitology* **125**, 75–78.
- Zarlenga, D.S., Rosenthal, B., La Rosa, G., Pozio, E. and Hoberg, E.P. (2006). An old genus learns new tricks: late tertiary colonization and speciation of *Trichinella* nematodes among Eutheria. *Proceedings of the National Academy of Sciences USA* **103**, 7354–7359.
- Zenker, F.A. (1871). Zur Lehre von der Trichinenkrankheit. *Deutsche Archiv für Klinische Medicine* **8**, 387–421.
- Zimmermann, W.J. (1970). The epizootiology of trichiniasis in wildlife. *Journal of Wildlife Diseases* **6**, 329–334.
- Zimmermann, W.J. (1971). Trichinosis. In: *Parasitic Diseases of Wild Mammals* (J.W. Davis and R.C. Anderson, eds), pp. 127–139. Ames: Iowa State University Press.
- Zimmermann, W.J. and Hubbard, E.D. (1969). Trichiniasis in wildlife of Iowa. *American Journal of Epidemiology* **90**, 84–92.

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Cover illustration: Scanning electron micrograph of metacyclic *Trypanosoma brucei* and the chemical structures of drugs used in the chemotherapy of human African trypanosomiasis (courtesy of Dr Laurence Tetley, University of Glasgow).
To illustrate the review by Barrett and Gilbert.

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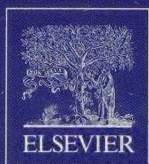
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